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THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

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AN ARTIFICIAL ANTIGEN WITH BLOOD-GROUP A SPECIFICITY.

W. T. J. MORGAN.

From the Biochemical Department of the Lister Institute, London.

Received for publication February 6, 1943.

THE investigations of Schiff (1930), Schiff and Weiler (1931), Landsteiner and Chase (1936), Meyer, Smythe and Palmer (1937), Goebel (1936), and of Landsteiner and Harte (1940) have shown that the substance present in commercial pepsin, peptone and hog gastric mucin, which possesses the serological characteristics of the specific blood-group A factor, is a polysaccharide-amino-acid complex. The substance is not antigenic but shows intense A specific serological activity, and is able to inhibit in extremely small amounts the agglutinating action of human anti-A (α)-agglutinin on group A erythrocytes. The predominantly polysaccharide nature of the serologically active complex suggested that it could be converted into an antigen showing A specificity by means of two simple methods which have already been described in detail (Morgan and Partridge, 1940; Partridge and Morgan, 1940). The methods were used originally to convert the specific, non-antigenic O polysaccharide of *Bact. shigae* gum acacia, cherry gum and kanten into antigenic complexes which gave rise to immune-body in the rabbit that was specific for the polysaccharide component of the artificial antigen (Morgan and Partridge, 1941; Partridge and Morgan, 1942). The investigation now to be described is an extension of this work on the conversion of non-antigenic polysaccharides into antigenic complexes, and the results show that the A specific substance in pepsin and hog gastric mucin can likewise be combined with the protein component of the O antigen of Shiga's bacillus, and that the resulting complex induces the formation of high titre anti-A immune serum in the rabbit. A short abstract, giving a preliminary account of this work, has already appeared (Morgan, 1941).

EXPERIMENTAL.

The A polysaccharide substance.—The gastric mucin polysaccharide used throughout this work was prepared from crude gastric mucin according to a method to be described shortly (Morgan and King, 1943), and possessed the following chemical and physical properties. It was soluble in 0·90 per cent. NaCl solution to give clear 0·1 per cent. solutions which showed $[\alpha]_{5461}+ 10°$ (C, 0·5). The substance contained 45·2 per cent. C, 6·7 per cent. H, 6·0 per cent. N (Dumas), 10·5 per cent. acetyl and 0·21 per cent. P in organic combination. The preparation was electrophoretically homogeneous in phosphate buffer (μ, 0·1) at pH 8·0 and showed a relative viscosity, η, of 2·86 (0·5 per cent. in 0·9 per cent. saline), compared with 0·9 per cent. saline as 1·00.

The 'Shiga' protein component.—A part of the preparation described by Partridge and Morgan (1942) was used to form the artificial A polysaccharide-'Shiga' protein complex. It showed $[\alpha]_{5461}- 50°$ (0·1 per cent. in 0·1 N NaOH), contained 54·2 per

cent. C, 6·5 per cent. H, 12·7 per cent. N (Dumas), 1·06 per cent. P (organic), and was insoluble in water within the pH range 1·0 and 6·5. The substance was electrophoretically homogeneous when examined in phosphate buffer (μ, 0·1) at pH 8·0.

The artificial antigenic complex.—Three different types of antigenic material were employed: (1) A preparation of A polysaccharide-'Shiga' protein complex made exactly as described earlier (Morgan and Partridge, 1940; Partridge and Morgan, 1940), by solution of the A polysaccharide (50 mg.) and 'Shiga' conjugated protein (10 mg.) in 5 ml. of neutral anhydrous formamide (m.p. + 2°). The final product formed an opalescent solution in water, from which only a small part of the complex could be thrown out by centrifugation at 3500 r.p.m. in the ordinary laboratory angle centrifuge. (2) A preparation of A antigenic complex formed from a saline solution of the same two components by simple pH adjustment (Morgan and Partridge, 1942). (3) In the presence of an excess (40–50 per cent.) of the protein component a considerable part of the complex formed in method (2) became insoluble when the pH of the solution was adjusted from alkaline reaction to 4·0–4·5. The precipitate formed under these conditions was collected, resuspended in fresh sterile saline, acidified to pH 4·5 with acetic acid, spun and again resuspended in fresh sterile saline. The exact composition of the insoluble complex, which was the third type of antigen employed, was not determined. It was established, however, that the final suspension of the insoluble precipitate gave a strong Molisch test for carbohydrate, and was capable under the test conditions and at a final dilution of 1 : 64 parts of saline of inhibiting the anti-A (α)-agglutinin activity of a human group B serum (α-agglutinin titre 1 : 192). This degree of activity indicated that about 10 per cent. of polysaccharide was present in the precipitated and resuspended material. The antigen preparations were kept at 0°, and fresh dilutions containing the required amount of substance were made in sterile saline immediately before use.

Immunization.—Selected rabbits that contained normal α-agglutinins were used for immunization. It has been shown by Dölter (1925), Witebsky (1928), Mai (1930), Schwarzmann (1936), Witebsky, Neter and Sobotka (1935), and Boyd (1939), that rabbits which contain a substance that is closely related to the human blood group A antigen in their tissues are unable to produce specific anti-A immune-body. Rabbits that possess natural α-agglutinins, on the other hand, do not contain the A antigen in their organs and are, therefore, able to produce A-antibodies. My experience supports this conclusion. In early experiments 16 rabbits were immunized with an artificial A antigen. Of these animals 6 only contained natural α-agglutinins in their serum, and these 6 animals alone responded and produced α-agglutinins of significant titre. All the antigen preparations studied and described in this paper were given by the intravenous route and the doses were spaced at 3- or 4-day intervals. Bleedings were taken 7–9 days after the last dose of antigen and the separated serum was immediately stored at − 20° without the addition of antiseptic.

Agglutination technique.—The titrations were carried out in small test-tubes. Increasing dilutions of serum (0·1 ml.), accurately measured with standard pipettes, were mixed with 0·1 ml. of 1 per cent. whole blood in saline. The degree of agglutination was read after the tubes had stood for 2 hours at room temperature. Fresh cells were used for each titration and the normal or immune-sera to be tested were heated at 56° for 20 minutes.

Precipitation reaction.—The tests were made by mixing 0·1 ml. of a 1 : 2 dilution of the test serum with 0·1 ml. of serial dilutions of the gastric mucin polysaccharide in normal saline. After 2 hours at 37° the tubes were kept overnight at 0° and examined while still cold by means of a hand-lens.

Haemolysis inhibition tests.—Haemolysis inhibition tests were set up as follows:

The immune serum was titrated to determine the minimal haemolytic dose (M.H.D.) against sheep cells in the presence of complement, and subsequently 2 M.H.D. of the serum contained in a volume of 0·5 ml. were mixed with increasing dilutions of the gastric mucin polysaccharide (0·5 ml.) and fresh guinea-pig complement (0·5 ml. of 1 : 20 dilution). After standing for 1 hour at 37°, 0·5 ml. of a 4 per cent. suspension of washed sheep cells in saline was added to each tube and the contents were thoroughly mixed. A control tube containing saline in place of the specific polysaccharide showed complete lysis after 20 minutes at 37°. The degrees of haemolysis were noted after the tubes had stood for 1 hour at 37° and overnight at 0–2°. The appropriate serum and complement controls were included in each inhibition test.

Anaphylaxis tests.—These tests were carried out exactly as described earlier (Morgan, 1932).

Immunization Results.

It was first necessary to establish that the purified gastric mucin A polysaccharide substance was itself not antigenic. For this purpose rabbits that possessed significantly high normal α-agglutinins were selected. For example, the sera of 5 rabbits used for testing the antigenicity of the gastric mucin preparation showed normal α-agglutinin titres of 1 : 128, 1 : 64, 1 : 128, 1 : 128 and 1 : 256 respectively. After 3 intravenous doses, each of 1 mg. of the preparation, blood samples were collected 7 days after the last dose and the sera, examined in the same order, showed α-agglutinin titres of 1 : 32, 1 : 256, 1 : 128, 1 : 64 and 1 : 64. Other rabbits were given 6 doses of 0·1 mg. of another preparation, but in no instance was a significant and specific immune response observed. Only about 20 per cent. of all rabbits examined possessed sera showing a natural α-agglutinin titre of 1 : 128 or above. The natural anti-B and anti-O titres of the sera of the selected animals were usually 1 : 32 or less. Occasionally the A titre of the serum of a normal rabbit will increase three to five-fold the original value as a result of immunization with the A polysaccharide alone, and for this reason an immune response giving rise to a serum showing a titre of less than five times the original value has been considered insignificant, and the material injected has been recorded as possessing no antigenic properties. On the other hand, the normal α-agglutinin titre is sometimes found to be reduced, as shown above, after immunization with the gastric mucin polysaccharide.

In order to show that the rabbits that possessed normal α-agglutinin and which had failed to respond when given the gastric mucin polysaccharide were, however, capable of producing strong anti-A sera, the animals were subsequently given 3 doses of the artificial antigen. Thus, in one experiment, the average α-agglutinin titre of the serum of three normal rabbits was 1 : 128, and after 3 doses of the A polysaccharide the average titre showed no significant change. The rabbits were then given 3 doses of an artificial A antigen; the average α-agglutinin titre increased to 1 : 16,384.

Table I shows the titration results obtained with five immune sera. In four of these the α-agglutinin titre after immunization was more than 100 times as high as the normal value. It will be noted that there is no significant rise in the anti-B immune-body. A total of 33 rabbits have responded in a similar manner after receiving 3 intravenous doses, each of 1·0 mg. or 0·10 mg. of one of the three types of artificial antigen.

Preparations of the A polysaccharide from commercial peptone or pepsin appear to be identical chemically with the A substance from gastric mucin. If the preparations have not been degraded during the manufacture of the peptone or pepsin they can be combined with the 'Shiga' protein and the resulting complex is again found to be antigenic. The results of the examination of the serum from two rabbits

TABLE I.—*Showing the Production of Anti-"A" Immune-body in Rabbits Inoculated with Three Doses each of 1·0 mg. of the Artificial Antigenic Complex.*

Rabbit number.	Type of antigen.	Source of A poly-saccharide.	Blood group of test-cells	Dilution of serum showing agglutination of erythrocytes.													
				1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	1:1024.	1:2048.	1:4096.	1:8192.	1:16,384.	1:32,768.
270	I	Pepsin	A_1	4	4	4	4	4	4	3	4	3	3	3	3	2	2
			B	2	1	1	0	0	0	0	..	..	..	..	..	..	..
			O	0	0	0	..	..	..	..	..	..	..	..	..	..	..
284	I	Gastric mucin	A_1	4	4	4	2	3	4	4	4	3	3	2	1	1	1
			B	3	3	2	1	0	0	0	..	..	..	..	..	..	..
			O	0	0	0	..	..	..	..	..	..	..	..	..	..	..
266	II	Pepsin	A_1	4	4	4	4	3	3	4	3	3	2	3	2	1	1
			B	3	3	3	2	1	0	0	0	..	..	..	..	..	..
			O	0	0	0	..	..	..	..	..	..	..	..	..	..	..
414	II	Gastric mucin	A_1	4	4	4	4	4	4	4	2	3	2	1	0	0	0
			B	3	3	1	1	0	0	0	0	..	..	..	..	..	..
			O	0	0	0	..	..	..	..	..	..	..	..	..	..	..
378	III	Gastric mucin	A_1	4	4	4	4	4	4	4	4	2	2	3	1	2	1
			B	4	3	3	1	1	0	0	0	..	..	..	..	..	..
			O	0	0	0	..	..	..	..	..	..	..	..	..	..	..

I, combination in aqueous solution at pH 9–11 and subsequent adjustment to pH 4·5; II, combination in anhydrous formamide and precipitation from solution by alcohol; III, as for I, but in presence of excess 'Shiga' conjugated protein.

1, A few groups of 2–3 cells; 2, larger groups with many free cells; 3, small clumps visible to the naked eye; 4, massive agglutination.

TABLE II.—*Showing the Examination of A Immune Rabbit Sera with Standard Red-cells of Different Sub-groups.*

Rabbit number.	Group of test-cells.	Dilution of serum giving agglutination.													
		1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	1:1024.	1:2048.	1:4096.	1:8192.	1:16,384.	1:32,768.
271	A_1	4	4	4	4	4	4	4	4	2	3	3	2	2	1
	A_2	4	4	4	4	4	4	3	4	2	2	1	1	0	0
	A_1B	4	4	4	4	4	4	4	2	3	3	2	1	1	1
	A_2B	4	4	4	2	3	3	3	3	2	2	1	1	0	0
	B	1	0	0	0	0	0	..	..	..	..	..	..	..	..
	O	0	0	0	0	0	0	..	..	..	..	..	..	..	..
478	A_1	4	4	4	4	4	4	4	3	3	3	1	2	1	2
	A_2	4	4	4	4	3	3	3	3	3	2	1	0	0	0
	A_1B	4	4	4	4	4	4	4	2	2	3	2	1	1	0
	A_2B	4	3	3	3	2	2	3	2	1	1	0	..	..	..
	B	2	1	1	0	0	0	..	..	..	..	..	..	..	..
	O	0	0	0	0	0	0	..	..	..	..	..	..	..	..

A_2B	4	3	3	3	2	2	3	2	1	1	0	..	..	..
B	2	1	1	0	0	0	..	..	..	..	..	..	..	..
O	0	0	0	0	0	0	..	..	..	..	..	..	..	..

TABLE III.—*Showing the Inhibition of Haemolysis of Sheep Cells in Different Types of Immune Sera (2 M.H.D.) by the Gastric Mucin A Polysaccharide.*

Rabbit serum prepared against—	Dilution of A polysaccharide.										
	1:10^3.	1:10^4.	1:10^5.	1:10^6.	1:2×10^6.	1:4×10^6.	1:8×10^6.	1:16×10^6.	1:32×10^6.	1:64×10^6.	Control.
Artificial gastric mucin A polysaccharide antigen	0	0	0	0	0	0	0	1	2	4	4
A red cells	0	0	0	0	0	0	1	2	3	4	4
Guinea-pig kidney . . .	4	4	4	4	4	4	4	4	4	4	4
Sheep red cells	4	4	4	4	4	4	4	4	4	4	4

0, No haemolysis; 1, trace of haemolysis; 2, definite haemolysis; 3, almost complete haemolysis; 4, complete haemolysis.

TABLE IV.—*Showing the Inhibition of Haemolysis of Sheep Cells in Immune Sera (2 M.H.D.) by Human Saliva.*

Nature of saliva.	Test sera.												
	Anti-A substance rabbit serum. Dilution of saliva substance.							Anti-A erythrocyte rabbit serum. Dilution of saliva substance.					
	1:10^4.	1:2×10^4.	1:4×10^4.	1:8×10^4.	1:10^5.	1:10^6.		1:10^4.	1:2×10^4.	1:4×10^4.	1:8×10^4.	1:10^5.	1:10^6.
Group A (active) .	0	0	0	tr.	1	2	.	0	0	0	0	tr.	3
Group A (inactive) .	4	4	4	4	4	4	.	4	4	4	4	4	4
Group B . . .	4	4	4	4	4	4	.	4	4	4	4	4	4
Group O . . .	4	4	4	4	4	4	.	4	4	4	4	4	4

immunized with artificial antigen made from A substance isolated from pepsin and protein component of the O antigen of *Bact. shigae* are given in Table I.

A more detailed examination of some of the anti-A sera was undertaken by Dr. G. L. Taylor, of the Galton Laboratory Serum Unit, Cambridge, to whom my thanks are due. Agglutination tests were made with A_1, A_1B, A_2, A_2B, B and O cells and the results obtained with anti-gastric mucin sera are given in Table II. Sera No. 271 and 478 were typical of most of the anti-sera to the gastric mucin polysaccharide-" Shiga " protein complex, the agglutination titre for A_1B cells being only slightly less than with A_1 cells. Similarly there was little difference between the A_2 and A_2B titres. The B and O titres of serum 271 were, however, exceptionally low. Dr. Taylor reported zones of partial agglutination followed by more complete agglutination at higher dilutions, but zones of this nature are largely omitted from the records of agglutination given in Table II, since our method of scoring is not so accurate as that used by Dr. Taylor and his associates. Only four degrees of agglutination are given in Table II. A few groups of about 2–3 cells seen under the microscope are scored as 1, larger groups with many free cells as 2, whereas small clumps visible to the naked eye are recorded as 3. Massive agglutination with practically no free cells is scored as 4. This coarser grading of degrees of agglutination, which has been substituted in Table II in place of Dr. Taylor's original values in order that the same degrees of agglutination should be recorded in all tables in this paper, largely eliminates the zones of partial inhibition, but there is no doubt that the zones recorded by Dr. Taylor do exist. This phenomenon has not been examined further.

The anti-sera gave definite precipitation with the gastric mucin polysaccharide. The amount of antigen-antibody precipitate was not large, but this was to be expected in view of the small amount of antigen and short course of immunization given to the rabbits. A quantitative determination of the amount of immune-body present in a pooled sample of an anti-A serum (α titre 1 : 4096) by the technique elaborated by Heidelberger, Kendall and Soo Hoo (1933) showed that 0·89 mg. of antibody protein was present per ml. of serum.

The results of haemolysis inhibition tests are shown in Table III. For purposes of comparison three other types of haemolytic immune serum were included in the tests. It will be seen that whereas an extremely small amount of the gastric mucin polysaccharide is able to inhibit the lytic action on sheep cells of 2 M.H.D. of the homologous immune sera or of an anti-A rabbit serum prepared against human A cells, the polysaccharide at a concentration as high as 0·1 per cent. fails to inhibit the haemolysis of the sheep cells by an anti-guinea-pig kidney (Forssman) or an anti-sheep cell serum. Under the same conditions a purified preparation of active human saliva of group A inhibited the lytic action of 2 M.H.D. of the anti-gastric mucin serum and of the anti-A-erythrocyte serum in much the same manner as the gastric mucin polysaccharide, whereas the saliva from a group A " non-secretor " or from a group B or O donor failed to cause any inhibition (Table IV).

Guinea-pigs that had received intraperitoneally 2–3 ml. of a potent A rabbit serum prepared against the artificial gastric mucin antigen failed to show any symptoms of anaphylaxis following an intravenous injection, 24 hours later, of quantities ranging from 0·1 mg. to 10 mg. of the homologous hapten. An active group A saliva given intravenously likewise failed to induce anaphylactic shock. The amount and potency of the antibody introduced was sufficient, assuming that no specific adsorption or neutralization of the immune-body occurred, to have given rise to a serum with an anti-A agglutinin titre of about 1 : 600, whereas the titre of the pooled serum from 12 guinea-pigs inoculated with the anti-A serum was found to be only 1 : 32. The A-titre of a pooled serum from a similar group of normal pigs was also 1 : 32. In order

to determine if guinea-pigs sensitized in a similar manner with an anti-O *Bact. typhosum* rabbit serum showed circulating antibody above the normal level the above experiment was repeated, and 24 hours after the intraperitoneal injection of the O serum, the animals were bled-out and the agglutinin titre of the serum against an O variant of *Bact. typhosum* was determined. The serum of the guinea-pig showed an anti-O-titre 30 times as high as the normal value. The amount of antibody retained in the serum represented 50–75 per cent. of the O agglutinin given intraperitoneally to the test animals.

DISCUSSION.

The results of the work described in this paper show that the non-antigenic polysaccharide complex that possesses blood-group A specificity, and is present in commercial pepsin and gastric mucin, can be combined with the protein component of the O antigen of *Bact. shigae*. In order that the A polysaccharide-'Shiga' protein complex shall be formed, however, it is essential that the polysaccharide used retains the high viscosity it possesses in its original, native state. The degraded form of the polysaccharide, which shows a much lower viscosity and decreased capacity to inhibit iso-agglutination, fails to combine satisfactorily with the protein and thus remains without appreciable antigenic properties. The complex derived from the undegraded polysaccharide is, however, strongly antigenic and gives rise to specific A immune-body when injected intravenously into rabbits that possess natural anti-A (α)-agglutinins. No significant increase in the natural anti-B or anti-O immune body occurs during immunization, whereas the titre of the anti-A immune substance shows a considerable rise and frequently attains a value as much as one hundred times the natural (normal) level.

The anti-A rabbit serum gave specific precipitation when incubated with the gastric mucin polysaccharide complex and with the saliva of " secretors " of group A, but not with the saliva from " non-secretors " of group A or with the saliva from individuals of groups B or O.

Guinea-pigs inoculated intraperitoneally with the anti-A rabbit serum failed to develop anaphylactic shock when subsequently given the unconjugated hapten intravenously. An A active saliva likewise failed to induce anaphylaxis. The most likely explanation for the failure is that the tissues of the guinea-pig, which are known to be rich in Forssman antigen, neutralize the anti-A immune-body injected intraperitoneally and thus protect the guinea-pig from specific sensitization. The amount and potency of antibody introduced should have given an antibody titre, as measured by agglutination of A cells, of about 1 : 600, whereas the average titre for the pooled serum of the 12 guinea-pigs used was only 1 : 32. A similar number of normal pigs gave a pooled serum showing the same A titre. The rapid rate of disappearance of the A agglutinin when compared with the retention of O *Bact. typhosum* antibody suggests that the anti-A substance was most probably neutralized *in vivo* by the A component of the Forssman antigen of the tissues.

The anti-A rabbit sera are strongly lytic for sheep erythrocytes in the presence of complement, and extremely small amounts of the purified A specific polysaccharide from hog gastric mucin or the A factor in the saliva of " secretors " can be detected by means of the haemolytic inhibition test. These active A substances fail to inhibit the lytic action of the anti-sheep red-cell or anti-guinea-pig kidney (Forssman) sera.

A number of the immune sera were titrated against human red-cells of the more common sub-groups. The majority of rabbits give rise to immune sera that agglutinate equally well with A_1 and A_1B erythrocytes and are, furthermore, found to be only slightly less active when tested against A_2 and A_2B cells. The immune-body is,

therefore, largely common α in character. Occasionally, however, anti-sera that show a significant difference in titre when tested with A_1 and A_2 cells are obtained. In certain instances this difference is quite large, and with one serum the agglutination titre against A_1 cells reached 1 : 32,768, whereas against A_2 cells the end-point of agglutination was only 1 : 1024.

It would be of considerable interest to examine the anti-A-immune body produced in response to immunization with an artificial antigen formed from the type specific polysaccharide of Pneumococcus (Type XIV), which has been shown by Goebel, Beeson and Hoagland (1939) to possess blood group A specificity, and the conjugated protein component of the O antigen of *Bact. shigae* or *Bact. typhosum*. The use of the purified A and B polysaccharide of human saliva as the specific hapten components of similar artificial antigenic complexes is being investigated.

The weak A reaction given by some specimens of blood group AB may cause the group to be diagnosed as B. Thomsen (1929) and Thomsen, Friedenreich and Worsaae (1930) have shown that in certain instances the B factor in AB blood is partially dominant to and, to some extent, obscures the A antigen. This tendency of the B factor to inhibit the serological reactions of the A component in erythrocytes of the type A_2B is even more pronounced and has recently been investigated in some detail by Taylor, Race, Prior and Ikin (1940). These workers showed that diagnostic sera of average potency might fail to detect the A component of an A_2B cell. It has now been shown that the powerful anti-A rabbit sera produced by means of the artificial gastric mucin polysaccharide antigen can be used to detect the A component of weakly reacting A_2B erythrocytes, and that agglutination of these cells occurs even with high dilutions of the serum. Although there has been no opportunity to examine the behaviour of A_3 or A_3B cells with the immune rabbit serum described in this paper, it seems probable that a preliminary diagnosis of this rare blood-group, which, in the latter instance, involves the detection of an A component in a cell appearing to show only B specificity, would be rendered easier and more certain if cells of this type, which react weakly or not at all with the usual anti-A test sera, were examined by means of the potent anti-A rabbit serum. Indeed, Wiener and Silverman (1941) have already employed potent anti-A sera, produced by immunizing rabbits with human A cells, for the detection of an A_3 blood-group, and more recently Taylor, Race, Prior and Ikin (1942) have shown that a very weakly reacting A blood-group antigen gave only feeble agglutination with the most powerful natural anti-A (α)-agglutinin they had encountered in the examination of 60,000 people. The α-agglutinin employed agglutinated A_1 cells to a dilution of 1 : 16,384, whereas with the weakly reacting cells, which most probably belonged to sub-group A_3B, the agglutination was only partial with the undiluted serum and the end-point was reached at a serum dilution of 1 : 16.

Immune anti-A sera obtained from animals may be used in place of human α-agglutinin in the technique of differential agglutination elaborated by Ashby (1919). In this technique a suspension of blood taken from a person of group A who has previously received a transfusion of group O blood is treated with a powerful anti-A serum, so as to agglutinate the A cells ; the unagglutinated O cells are then counted, and by repeating the test at intervals after transfusion, their survival in the recipient's circulation may be estimated. It has been observed, however, that human anti-A sera sometimes fail to bring about sufficiently complete agglutination of the A cells, particularly when the recipient belongs to sub-group A_2, and an accurate estimate of the number of O cells present is therefore no longer possible because of the large number of unagglutinated A cells. In such instances Wiener (1939) has recommended the use of immune animal serum, and the rabbit immune sera described in this paper have likewise been found to give very complete agglutination and have permitted an

accurate estimation of survival of O cells to be made. Recently, Dr. P. L. Mollison has reported that the agglutination, by means of the A specific anti-gastric mucin sera, of all but 0·04–0·40 per cent. of total A cells has frequently been attained. When immune animal sera are used for this technique however control tests must be set up with O cells to make sure that no non-specific agglutination is occurring. It is also advisable to test serial dilutions of the serum, because the formation of zones wherein agglutination is absent or incomplete is known to occur and the most complete agglutination is sometimes not obtained with the undiluted serum.

SUMMARY.

(1) The formation of an antigenic complex from the non-antigenic A polysaccharide-amino-acid complex derived from commercial pepsin or hog gastric mucin and the conjugated protein component of the specific O somatic antigen of *Bact. shigae* is described.

(2) The artificial antigen induces the formation of extremely potent immune-body in the rabbit. The immune serum agglutinates human erythrocytes belonging to blood group A, but fails to agglutinate those of groups B or O.

(3) The anti-A sera cause the haemolysis of sheep red cells in the presence of complement. The haemolysis is inhibited by the homologous hapten (gastric mucin A polysaccharide) and by the blood group A polysaccharide in the saliva of individuals secreting this factor. The saliva of non-secretors belonging to group A and the saliva of individuals of group B or O fail to cause any haemolysis inhibition.

(4) The possible use of these high titre immune sera for the detection of the weakly reacting erythrocytes, such as those of blood group A_2B, A_3 and A_3B is discussed and their use as reagents in the technique of differential agglutination is indicated.

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THE PRODUCTION AND TREATMENT OF EXPERIMENTAL PNEUMOCOCCAL HYPOPYON ULCERS IN THE RABBIT.

J. M. ROBSON AND G. I. SCOTT.

From the Department of Pharmacology, University of Edinburgh.

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IN previous investigations it has been shown that lesions of the rabbit's eye produced by *B. pyocaneus* and *Staphylococcus aureus* can be successfully treated by the local application of various chemotherapeutic substances (Robson and Scott, 1942, 1943). These organisms were chosen for investigation because they are not easily affected by chemotherapy, and hence their successful treatment provides convincing evidence of the value of the local applications used.

Of great interest and importance from the point of view of the human eye is the lesion, ulcus serpens, produced by the pneumococcus, and it seemed therefore desirable to determine whether experimental pneumococcal ulcers would also respond to chemotherapeutic treatment.

Attempts have previously been made to produce pneumococcal lesions of the eye of the rabbit in order to study the value of substances locally applied. Thus Ginsberg and Kaufmann (1913) produced progressive infiltration by the injection into the cornea of rabbits of blood obtained from animals dying from a pneumococcal septicaemia, and studied the effects on the bacterial content of these lesions of local treatment with quinine derivatives. As far as we know, however, no pneumococcal lesions similar in type to those seen in the human cornea have been successfully produced and studied in the rabbit or any other animal, and it seemed to us particularly desirable to obtain, if possible, lesions of this type for the investigation of various forms of therapy.

We tried various types of pneumococci, and either applied them to the scarified cornea or injected them into the cornea. But, though we succeeded in producing infiltration similar to that described by Ginsberg and Kaufmann, no ulceration developed. We then put the problem to Dr. W. M. Levinthal, of the Royal College of Physicians Laboratory, Edinburgh, and he suggested the use of a pneumococcus type 19 and supplied us with a strain of the organism. In preliminary experiments it was found that the injection of this organism into the cornea of rabbits rapidly led to ulceration, which was frequently accompanied by the formation of hypopyon, and by iritis. This strain was therefore used in all our subsequent investigations.

METHODS.

All experiments were performed on mature rabbits of both sexes and various breeds.

The corneal injections were performed with the technique previously described (Robson and Scott, 1943). A 6 to 15 hours' culture of pneumococcus (type 19) in plain broth was used in all experiments. The culture was injected to form a small bleb in the superficial part of the cornea. Great care was taken to ensure that the lesions were as equal as possible in both eyes of any one animal.

The first treatment was applied at various intervals (1, 6, 12 and 24 hours) after

inoculation and was continued at hourly intervals for some 36 hours. Frequent treatment was thought advisable in order to maintain an adequate concentration of the chemotherapeutic substance in the tissues of the eye and particularly in the cornea (see Robson and Tebrich, 1942). Thereafter, treatment was given hourly during the day. The total duration of the treatment did not, as a rule, last for more than 5 days. Whenever treatment was applied to an eye a similar quantity of normal saline was applied to the other eye (i.e. the control).

The chemotherapeutic agents used were :

1. *Penicillin.*—A solution of the barium salt containing some 65 Oxford units per c.c. was, before use, converted into the sodium salt by the addition of the necessary amount of solid sodium sulphate, which precipitated the barium.

2. *Sodium sulphacetamide.*—This was supplied to us as a 30 per cent. solution.

3. *Sodium sulphapyridine* used as a 30 per cent. solution in saline.

4. *Tyrothricin.*—The powder was suspended in saline (1 mg. per c.c.). This suspension was vigorously shaken before every treatment.

The penicillin and tyrothricin solutions were stored in the refrigerator when not in use.

The eyes were examined daily during the first few days and thereafter at less frequent intervals. Detailed records were kept of the condition of the eyes, as described in a previous report (Robson and Scott, 1943). The eyes were usually photographed at 48 or at 72 hours and at intervals throughout the progress of the lesion.

RESULTS.

There is considerable variation in the rate of development of the lesions. This variation is, however, much less in a particular group of animals done at one and the same time than amongst groups of animals done on different dates, suggesting that the variation between the different groups is due to differences in the virulence of the cultures of the organisms. The lesion described below is of the more severe type which occurs in the majority of animals.

Twenty-four hours after inoculation the cornea shows slight infiltration at the site of inoculation, which is surrounded by an area of oedema. There is also greyish-white infiltration at the margin of the affected area, and its central part practically always stains with fluorescein. In some cases ulceration is already developing, even at this early stage. There is also moderate congestion and slight oedema of the conjunctiva with ciliary congestion and some iritis.

At 48 hours definite ulceration is developing, and the epithelium has desquamated over the whole of the area of inoculation and usually beyond it. There is subepithelial infiltration beneath the margin of the desquamated area, as typically seen in pneumococcal ulcers of the human cornea. The ulcerated area is surrounded by a zone of oedema, and the iritis is now considerably more severe. Conjunctival discharge is usually only slight in amount, and this applies throughout the whole course of the lesion. Hypopyon is only rarely present at this stage.

On the third day the centre of the cornea is occupied by a deep spreading ulcer, usually about 4 mm. in diameter. In many cases a moderately large hypopyon is present. The iritis is now even more severe, and has become plastic in type. The appearance of the ulcer is very similar to the illustration given by Duke Elder (1938) of a pneumococcal ulcer of the human cornea.

During the next few days there is a spread of the ulcerative process both in depth and extent. The most marked feature is the degree to which infiltration occurs into the surrounding cornea beneath the overhanging edge of the original area of ulceration. The ultimate picture at this stage, therefore, is of a central area of actual ulceration

open to the surface, surrounded by a zone of massive cellular infiltration of the substance of the cornea. The hypopyon is increasing in amount, although it becomes obscured in severe cases by the corneal lesion. Luxuriant ingrowth of vessels from the limbus towards the ulcerated area occurs towards the end of the first week.

In some cases, however, there is little spread of the lesion beyond the 48 hours' stage, with slight or no hypopyon.

The lesion shows a natural tendency to heal during the second week. In the majority of cases this leaves an area of dense scarring, about 5 mm. in diameter in the centre of the cornea ; this is surrounded by an area of haziness through which the vessels run from the limbus towards the centre. In some cases the central lesion may be completely surrounded by a thick fleshy vascular pannus. Very occasionally perforation of the cornea occurs.

As already stated above, there is considerable individual variation and, in some animals, the lesion is less severe, and finally consists of a moderately dense central scar about 4 mm. in diameter with a leash of vessels growing in towards it. In some cases these vessels almost completely disappear as the lesion heals.

Effects of Treatment.

About one-third of the inoculated animals died from a general septicaemia, usually within the first three days, and cultures of the heart blood revealed the presence of numerous pneumococci.

Treatment with penicillin started at 1 hour (experiment on 12 rabbits, of which 4 died during the first 3 days).

Treatment started at this stage completely prevented ulceration, except in one animal.

In 7 out of the 8 animals which survived more than 3 days, the course of the lesion in the treated eye was as follows : Twenty-four hours after inoculation there was faint greyish infiltration at the site of inoculation about 2 × 2 mm. in size, with very slight surrounding oedema. In addition there was slight congestion of the palpebral conjunctiva, but no iritis and no ciliary injection. There was no staining with fluorescein in any of the eyes. On the 3rd day the eye was completely clear and showed no abnormality apart from a minute superficial scar at the site of the inoculation. In the 8th animal the condition up to 48 hours was as in the other 7, but at 72 hours there was a definite increase in the amount of oedema surrounding the small corneal infiltrate and the cornea also stained slightly with fluorescein. During the next few days a small ulcer developed with ingrowth of vessels from the limbus, but by the end of the second week healing had occurred without further spread, leaving a thin superficial scar some 3 × 2 mm. in size. In the four animals which died, the treated eyes showed only a little infiltration at the site of inoculation. In all the 12 control eyes a severe lesion developed. A well-defined ulcer was present at 24 hours and was associated with ciliary congestion. Hypopyon developed in 6 out of 8 animals which survived, and all were left with dense vascularized scars.

Treatment with penicillin started at intervals longer than 1 hour.

The treatment with penicillin started at longer intervals than one hour after inoculation, or with other drugs even when started at one hour after inoculation, did not completely prevent the development of corneal lesions, although definite effects were often observed. In order to express the results quantitatively the following method of classifying the lesions was adopted. The lesions were divided into five grades of severity, namely :

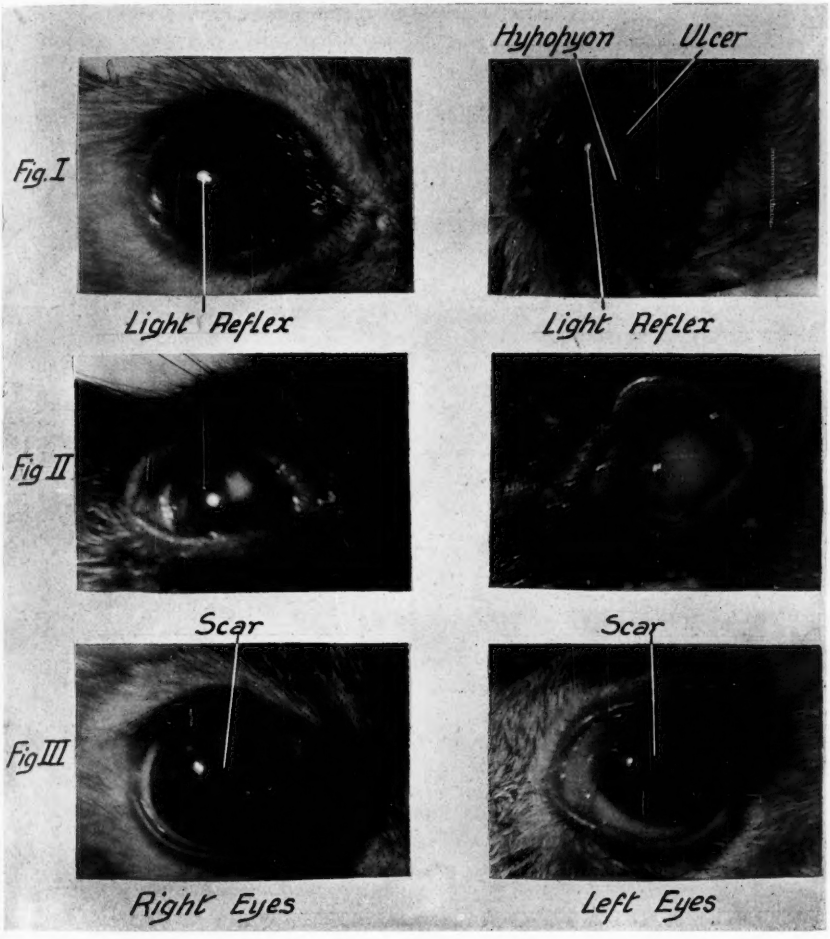

FIG. 1.—Illustrating a typical hypopyon ulcer on the sixth day after inoculation (left eye) and the effect of treatment with penicillin started 12 hours after inoculation (right eye) (Rabbit 952).

FIG. 2.—Illustrating the effect of treatment with penicillin started 24 hours after inoculation. The right eye received treatment (Rabbit 945).

FIG. 3.—Illustrating the lesions left in the eyes a considerable time after inoculation (Rabbit 908 on the 43rd day after inoculation) Right eye: Treatment with 30 per cent. sodium sulphacetamide started one hour after inoculation. Left eye: Control.

(1) No ulceration and ultimate scar small and superficial and limited to the site of inoculation.

(2) Small ulcer or infiltration with oedema at site of inoculation but without spread, and resulting in a thin, superficial scar not greater than 2·5 × 2·5 mm. in size.

(3) Development of a deep well-defined ulcer, up to about 4 × 4 mm. in size. Hypopyon did not usually develop in lesions of this severity.

(4) Ulcer larger than type 3, and nearly always associated with hypopyon and with ingrowth of vessels from the limbus towards the affected area.

(5) Gross lesion involving most of cornea and resulting in dense and extensive vascularized scarring.

It should be noted that lesions 1 and 2 resulted in only slight scarring which would not seriously impair the function of the eye, whilst the more serious lesions produced dense scars of considerable size, which would greatly interfere with vision.

The results for treatment with penicillin started respectively at 1, 6, 12 and 24 hours are shown in Table I. It will be seen that even when started at 12 hours the treatment had a beneficial effect. When started 24 hours after inoculation the treatment was less effective, although still of some value.

TABLE I.—*Showing the Effect of Treatment with Penicillin, Started at Various Periods after Inoculation.*

	Number of animals.	Corneal lesions. Very slight. (1).	Slight. (2).	Moderately severe. (3).	Severe. (4).	Very severe. (5).	Interval between inoculation and beginning of treatment.
Treated eyes	8	7	1	0	0	0	1 hour.
Control ,,		0	0	3	5	0	
Treated ,,	9	1	2	5	1	0	6 hours.
Control ,,		0	0	0	6	3	
Treated ,,	11	1	10	0	0	0	12 ,,
Control ,,		0	0	3	7	1	
Treated ,,	14	0	8	5	0	1	24 ,,
Control ,,		0	0	7	3	4	

Sodium sulphacetamide.

When treatment was started at 1 hour after inoculation it was effective in every case in preventing the development of more than a very small ulcer. Obviously,

TABLE II.—*Showing the Effect of Treatment with 30 per cent. Sodium Sulphacetamide, Started at Various Periods after Inoculation.*

	Number of animals.	Corneal lesions. Very slight. (1).	Slight. (2).	Moderately severe. (3).	Severe. (4).	Very severe. (5).	Interval between inoculation and beginning of treatment.
Treated eyes	9	1	8	0	0	0	1 hour.
Control ,,		0	0	9	0	0	
Treated ,,	5	0	2	3	0	0	6 hours.
Control ,,		0	1	0	3	1	
Treated ,,	6	0	1	5	0	0	12 ,,
Control ,,		0	0	5	0	1	
Treated ,,	5	0	0	5	0	0	24 ,,
Control ,,		0	0	1	4	0	

however, the results were not as good as with penicillin, in which ulceration was completely prevented in all but one animal. When treatment was delayed until 6 hours after inoculation the effect was much less, and at 12 hours and 24 hours the effect of treatment was negligible (Table II).

Sodium sulphapyridine.

Since sulphapyridine has been widely and effectively used in the treatment of pneumococcal pneumonia, it was expected that the results in the local treatment of pneumococcal lesions would be good. In one experiment, done on 6 rabbits, 4 survived for more than 3 days. In these the treatment, started one hour after inoculation, was definitely of value, though ulceration was not prevented (Table III). In one of the eyes treated with sodium sulphapyridine, particles of solid were deposited in the cornea and made the total lesion a good deal worse, though the infective lesion was only slight. The repeated instillation of the alkaline solution also appeared to produce some irritation of the conjunctiva. In view of these results the experiments with sodium sulphapyridine were not continued.

TABLE III.—*Showing the Effect of Treatment with Sodium Sulphapyridine and with Tyrothricin (Gramicidin) Started One Hour after Inoculation.*

	Number of animals.	Corneal lesions. Very slight. (1).	Slight. (2).	Moderately severe. (3).	Severe. (4).	Very severe. (5)	Treatment.
Treated eyes	4	0	4	0	0	0	Sodium sulphapyridine.
Control ,,		0	1	2	1	0	
Treated ,,	5	0	3	1	1	0	Tyrothricin.
Control ,,		0	1	3	1	0	

Tyrothricin.

In view of the fact that gramicidin, the main active constituent of tyrothricin, has been shown to produce a highly beneficial action in experimental infections of mice with pneumococcus (Hotchkiss and Dubos, 1940), this substance was tried in the local treatment of the pneumococcal lesions. The experiment was performed on 6 rabbits, of which 5 survived for more than 3 days. The treatment was started one hour after inoculation. It possibly produced slight benefit, though ulceration was not prevented in any of the treated eyes (Table III).

DISCUSSION.

To the best of our knowledge this is the first time that an experimental corneal ulcer of hypopyon type has been produced by inoculation with the pneumococcus. In many respects the lesion produced is very similar to the typical pneumococcal ulcer of the human eye. Although, however, hypopyon develops in all cases in which severe ulceration occurs, ulcers of moderate size (about 3 × 3 mm.) may develop without being associated with an hypopyon. Moreover, whilst the edges of the ulcer in the rabbit are characteristically undermined as in the human subject, the lesion often spreads at several points simultaneously rather than at one spot, as is the case in the typical human "ulcus serpens." The lesion is associated, as in man, with a severe plastic iritis.

As is to be expected, the lesion develops much more rapidly in the rabbit than in

the human subject, and a deep well-developed ulcer is usually present by the third day after inoculation. It is to be noted, however, that human ulcers probably arise after a minute abrasion and the introduction of probably comparatively few organisms, whereas in these experiments large numbers of highly virulent organisms were injected into the cornea.

Whilst a severe ulcer develops with rapidity in the rabbit, the lesion tends to reach its maximum towards the end of the first week ; thereafter the ulcer does not, as a rule, spread further, but becomes gradually epithelialized as vascularization and fibrous changes occur. Perforation occurred only very occasionally.

The development of this typical progressive lesion could be beneficially influenced by local treatment with certain chemotherapeutic substances. The results obtained, however, emphasize the importance of commencing the treatment as early as possible, and show that delay of even a few hours may make a lot of difference to the lesion which ultimately results. This is in agreement with the findings previously obtained on lesions of the eye produced by *B. pyocyaneus* and *Staphylococcus aureus* (Robson and Scott, 1942, 1943).

The beneficial effects obtained with sodium sulphacetamide were rather unexpected, since this sulphonamide is not usually considered as being of any great value in the treatment of pneumococcal infections. On the other hand, this substance does penetrate very rapidly into the cornea, producing high concentrations, and this may be the explanation of its effectiveness in these lesions.

The most striking results were, however, obtained in the eyes treated with penicillin. Not only was early treatment (i.e. one hour after inoculation) capable of preventing the development of any ulceration in all but one of the animals, but, when the treatment was delayed for 24 hours, quite appreciable beneficial effects were still produced. These results, like those previously obtained in the treatment of experimental staphylococcal lesions of the cornea (Robson and Scott, 1943), are evidence of the great potential value of penicillin in the local therapy of infected lesions.

The striking results obtained experimentally in the rabbit are not likely to be seen clinically in the human subject, since there treatment will not usually be instituted until after the development of a definite lesion. It must be remembered, however, that the lesion in the human eye develops more slowly than the experimental lesion in the rabbit's eye, so that treatment may be expected to be effective at longer intervals after the beginning of the lesion in the human than in the rabbit's eye. This applies especially to penicillin, which seems obviously worthy of clinical trial.

Chemotherapeutic substances may prove to be of great value in the prevention of corneal sepsis following various types of injuries since, under those circumstances, they can be applied at a very early stage. The use of sodium sulphacetamide in the prevention of hypopyon ulcers in eye injuries of miners has, indeed, already been described by Dickson (1942).

SUMMARY.

1. The injection of a Type 19 pneumococcus into the cornea of the rabbit is followed by the development of an ulcer, frequently associated with hypopyon, and very similar in type to the pneumococcal hypopyon ulcers seen in the human cornea.

2. Definite beneficial effects upon the development of such lesions were produced by the local application of penicillin and of 30 per cent. sodium sulphacetamide. The effects with penicillin were especially striking. Tyrothricin was of little value.

3. Delay in treatment with sodium sulphacetamide for more than 6 hours greatly decreased the value of the treatment. With penicillin, however, quite appreciable effects were still obtained when the treatment was delayed for 24 hours.

We are very grateful to Dr. Levinthal for his valuable suggestions and help in this work. We are also indebted to the " W. H. Ross Foundation (Scotland) for the Prevention of Blindness," who have defrayed the expenses of this investigation ; to Sir Henry Dale, who kindly gave us some of his supply of tyrothricin ; to Dr. Trevan, of the Wellcome Research Foundation, who kindly supplied us with penicillin made by Dr. Pope and Miss Stevens ; and to Mr. Edwards, of British Schering, Ltd., who kindly supplied us with sodium sulphacetamide (Albucid soluble).

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A PURE STRAIN OF ROUS SARCOMA CELLS.

E. TENENBAUM.

From the Department of Experimental Pathology, The Hebrew University (Cancer Laboratories), Jerusalem.

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THE usual method of cultivation of Rous chicken sarcoma is that of Fischer (1925), who keeps the sarcoma cultures permanently alive and virulent by the regular addition of normal tissue to the tumour-cell colonies. Fischer assumes that the added normal tissue furnishes the necessary mechanical support to the outwandering sarcoma cells, which by liquefying the plasma clot deprive themselves of such support. According to him the added tissue is digested and assimilated by the neoplastic cells.

Besides mechanical and nutritive functions the added normal tissue has another significance. The causative agent, present in the sarcoma cell cultures, combines with the normal cells and provokes their malignant transformation (Ludford, 1937 ; Halberstaedter and Doljanski, 1939 ; Halberstaedter, Doljanski and Tenenbaum, 1941). Thus in these cultures apart from the multiplication of the sarcoma cells a permanent process of cancerization of the added normal tissue takes place.

It is evident that sarcoma cultures maintained according to Fischer's method cannot be considered as pure colonies of sarcoma cells ; they contain malignant cells, normal cells and elements involved in a process of gradual cancerization. Most investigations dealing with sarcoma cells *in vitro* were carried out of necessity on such very heterogeneous cell material, ill adapted to either morphological or physiological study. It seemed therefore desirable to attempt the culture of Rous sarcoma cells *in vitro* without addition of normal tissue. As we show below, Rous sarcoma cells can be kept easily under these conditions as a pure culture over a long period of time (4–6 months).

MATERIAL AND TECHNIQUE.

Our sarcoma strains originated from sarcoma cultures cultivated through 520 passages according to Fischer's method. The cultures were carried out by the standard

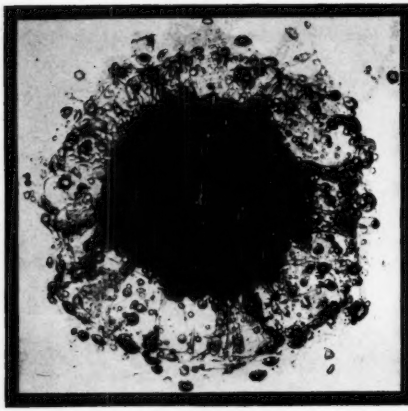

FIG. 1.

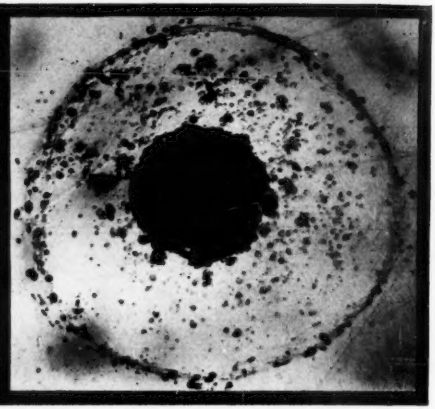

FIG. 2.

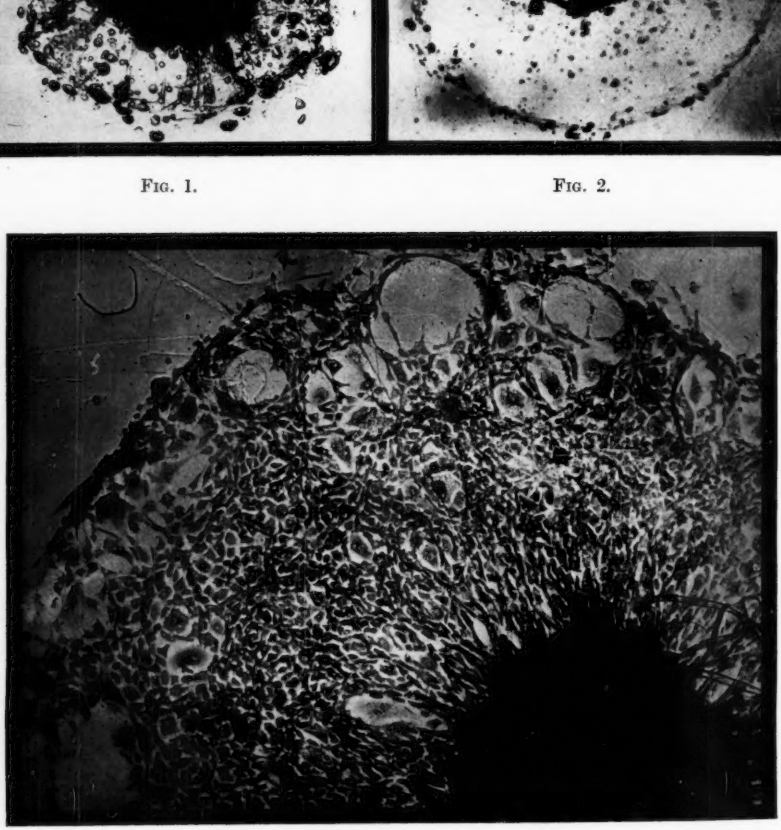

FIG. 3.

FIG. 1.—Pure Rous sarcoma culture 18th passage, living. × 71.
FIG. 2.— „ „ „ 20th passage, living. × 59.
FIG. 3.— „ „ „ 31st passage. Giemsa stain. × 50.

Tenenbaum.

hanging drop method in equal parts of fowl plasma and diluted embryonic extract. Passages were made every second or third day.

RESULTS.

The behaviour of colonies of sarcoma cells when cultivated for several passages without addition of normal tissue is as follows: Soon after transferring, numerous spindle-shaped and round cells begin to migrate from the original fragment. They form at first a compact growth area composed chiefly of spindle cells and scattered round cells. This outgrowth pattern is usually not maintained for long. The strong proteolytic activity of sarcoma cells interferes with the maintenance of a continuous growth area. Very soon after transferring the fibrin clot undergoes liquefaction, the growth area is split up, and the cells are separated one from another. As a consequence of this development we find, as early as 2 to 24 hours after transferring, the original fragment surrounded by a large liquefied area, occupied by numerous single rounded cells. The area of liquefaction is always outlined by a ring composed chiefly of connected spindle cells. At some places in this ring increased proliferation of the cells may result in the formation of nodule-shaped swellings, which often attain a considerable size. During the process of cultivation the central piece becomes completely rounded and acquires a glassy appearance. As a rule it presents itself as a semi-transparent lens-like body. The liquefied plasma around the original fragment takes on a viscous consistency, and a vigorous multiplication of free rounded cells takes place in this slimy medium.

The colony of sarcoma cells cultivated without addition of normal tissue possesses a peculiar and characteristic appearance which is maintained over a prolonged time (Figs. 1 and 2). The multiplication of cells is vigorous, and the cell colonies could be divided at every second or third passage. Thus it is possible to obtain in a short time from a few initial Rous sarcoma cultures large numbers of cell colonies.

The characteristic appearance of sarcoma-cell cultures depends to a high degree on the presence of an adequate amount of embryonic extract in the culture medium. When cultivated without or with a small quantity of embryonic extract the cultures show an entirely different aspect. As liquefaction does not occur under these conditions, the spindle cells that proliferate from the central piece are able to form an extensive continuous growth area. This is to be seen after 24–48 hours' development as a rich layer consisting of a network of connected spindle and star-like cells, the majority of which are flattened (Fig. 3). After transferring to a medium containing sufficient amount of embryonic extract, such a culture reverts to the typical pattern.

Cultivation of sarcoma cells without addition of normal tissue is possible over a long period of time. We have kept several cell strains in culture over a period of 4–6 months each (40–50 passages). It has not proved possible, however, to maintain such cell strains for longer than this time.

All our efforts to obtain a permanent pure strain of Rous cells were without success, and we are inclined to conclude that this objective is not realizable. The obstacles in the way of this undertaking were not of a technical nature but intrinsic. During the first 30 passages the sarcoma-cell colonies grow well and active multiplication of cells occurs, but in later passages changes set in which interfere with a normal development of the cultures. After the period of undisturbed growth the vigorous development ceases and the cultures become gradually poorer in cells. This process ultimately leads to the death of the cell colonies. We propose to describe elsewhere the peculiar pathological changes which constantly develop in sarcoma cells after a certain number of passages. These changes interfere with permanent culture and render it impossible.

The sarcoma cells in pure culture maintain their malignant properties during the entire period of cultivation. Inoculation of such colonies into chickens always produced typical tumours. Altogether we have inoculated a total of 16 cultures, ranging in age from 9–30 passages, and in all cases obtained a positive result. The tumours were accompanied by metastases.

In conclusion we may mention that Carrel (1912), in his earlier tissue-culture investigations, made attempts to cultivate sarcoma-cell cultures. He was able to maintain the sarcoma-cell strains for 46 days. At the end of this time the cultures died " without any apparent cause."

SUMMARY.

The cultivation of Rous sarcoma colonies as a pure cell strain is reported. The pure sarcoma-cell cultures can be maintained *in vitro* for a period up to six months. During a prolonged time the cells proliferate vigorously, but ultimately they undergo pronounced alterations, progressively degenerate and die. The aspect and behaviour of pure Rous sarcoma-cell strains is described.

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THE MECHANISM OF RED BLOOD CELL DESTRUCTION.

B. G. MAEGRAITH, N. H. MARTIN and G. M. FINDLAY.

From a Military Laboratory.

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It is generally believed that the reticulo-endothelial system is responsible for destruction of red blood cells in the normal body, but the exact factors controlling the rate and extent of this destruction are not accurately known. It occurred to one of us (B. G. M.) that lysis of erythrocytes might be a biological process associated with the destruction of the red cell membrane or substance by an enzyme, in a manner similar to the break-up of certain bacterial bodies in the presence of lysozyme, where one particular fraction is broken down by the specific enzyme (Epstein and Chain, 1940).

A series of experiments was therefore designed to investigate red cell destruction from this angle. The technique and results of these experiments are described in this communication.

Experimental results so far demonstrate the presence of a specific lytic agent in animal tissues. Although complete proof of its nature is not yet available, it is considered that this lytic agent is probably an enzyme.

The existence of substances capable of inhibiting the action of the lytic agent has also been demonstrated both in the tissues and in the blood serum. It is suggested that the balance between the lytic agent and the inhibitory factor controls the degree

of lysis obtaining in the tissue at any given time, and that alteration of this balance by extraneous factors will result in either increase or decrease of erythrocyte destruction. Thus a reduction in the inhibiting action would give rise to increased lysis, and vice versa.

The quantitative distribution of the lytic agent in the tissues has not yet been worked out, but experiments so far indicate that lytic power is *not* confined, as is so frequently suggested, to tissues generally considered to be rich in reticulo-endothelial cells.

Our experiments have been carried out under Service conditions, and it has been impossible to complete them with the apparatus available. Nevertheless, the results at this stage appear such as to warrant presentation, since they may give a pointer to the mechanism of lysis in blackwater fever and other haemolytic anaemias.

Experiments on these lines are continuing on blackwater fever and chronic malarial anaemias.

METHODS.

Preparations of Materials.

Glass-ware.

All glassware should be passed through the following process for thorough sterilization:

First wash in warm soapy water and rinse through with tap water. Secondly, wash in 1 per cent. hydrochloric acid, and clean in distilled water. Thirdly, wash in industrial spirit, and again cleanse in distilled water. Finally, dry and sterilize at 25 lb. pressure in an autoclave for one hour.

Red blood cells.

In the various experiments one of the following was used as a suspending medium: (*a*) 0·85 per cent. sterile saline; (*b*) 0·85 per cent. sterile saline, containing 5 per cent. glucose; and (*c*) 0·85 per cent. sterile saline buffered to a pH of 7·1 with potassium acid phosphate/disodium phosphate.

Blood was obtained from monkeys by cannulation of the carotid under pentathal or urethane anaesthesia, the blood being defibrinated with glass beads. Blood was obtained from guinea-pigs by direct cardiac puncture through the exposed muscle wall under ether anaesthesia.

5 ml. of sterile defibrinated blood were suspended in 20 ml. of the suspending fluid and the cells spun down at 2000/2500 revolutions. The supernatant fluid was drained off and three or more further successive washings carried out until the supernatant was clear. The final cell suspension was adjusted to a concentration of 500,000 cells per cubic mm. (equivalent to a 10 per cent. cell suspension.)

Tissues.

Monkeys were exsanguinated by the technique described by Dreyer and Ray (1910), using buffered saline for the final washing. Portions of the tissues required for the experiment were removed with full aseptic precautions. The tissues were sliced into small pieces, approximately 4 mm. square by 2 mm. thick, and stored in sterile Petri dishes at 2° C. The delay between excision and use was never more than two hours.

Guinea-pigs were exsanguinated by bleeding. Tissues were then removed and sliced, as in the case of monkeys.

Washed tissue was prepared by washing the sliced tissue in 6 in. Petri dishes in two changes of suspending fluid at 0° to 2° C., each washing taking half an hour.

Serum.

This was obtained in the usual way and diluted to the required concentrations with the saline solution used for suspending the corpuscle.

Potassium cyanide and mercuric chloride solutions.

These were prepared from pure chemicals dissolved in the suspending fluid.

Experimental Details.

The standard experimental tissue : : red cell suspension system.

The standard system was set up as follows :

Tubes 1 *and* 2 : 1 c.c. of red cell suspension with 1 c.c. of suspending fluid = 5 per cent. final red blood cell suspension.

Tubes 3 *and* 4 : 1 c.c. of red blood cells + 1 c.c. of suspending fluid + 1 slice of washed tissue.

Tubes 5 *and* 6 : 1 c.c. of red blood cells + 1 c.c. of the required dilution of inhibiting agent (serum, potassium cyanide or mercuric chloride) + 1 slice of washed tissue.

Tubes 7 *and* 8 : 1 c.c. of red blood cells + 1 c.c. of suspending fluid + 1 slice of unwashed tissue

Tubes 9 *and* 10 : 1 c.c. of red blood cells + 1 c.c. of suspending fluid + 1 slice of washed tissue previously heated at 80° C.

In such a system, after incubation at 37° C., complete haemolysis occurred consistently only in tubes 3 and 4. Haemolysis in tubes 5 and 6 was determined by the dilution of inhibitor.

An example of an actual experiment set up as above is as follows :

System 1. *Tissue :* Guinea-pig lung.
,, 2. *Red cells :* Guinea-pig red cells ; washed in glucose saline and prepared in 10 per cent. suspension.
,, 3. *Serum :* Taken from N.H.M.

		24 hours.	36 hours.
Tubes 1 *and* 2 :	Red cells + saline	0	0
,, 3 ,, 4 :	Red cells + saline + washed lung . .	PL.	L.
,, 5 ,, 6 :	Red cells + serum 1/100 + washed lung .	0	0
,, 7 ,, 8 :	Red cells + serum 1/150 + washed lung .	0	0
,, 9 ,, 10 :	Red cells + saline + unwashed lung .	0	0

Read after 24 hours and 36 hours. Incubation at 37° C.

Note.—PL. = partial lysis ; L. = lysis.

Extraneous factors affecting constancy of results.

(1) The need of strict asepsis during bleeding and removal of tissues has already been stressed together with the avoidance of bacterial contamination during the setting up of the tissue : : red cell suspension systems. Under the conditions of a tropical climate it was found essential to set up all experimental systems and controls at least in duplicate.

(2) Traces of chemical impurities in or on the glassware due to inadequate washing were at first a further cause of irregularities in results. To get over this, 40 clean uniform test-tubes were selected, subjected to rigorous cleaning, and then used exclusively for all experiments, of course with the necessary cleaning and sterilizing before each new experiment.

(3) It was also observed that the suspending media had some effect on the stability of the system. The pH had to be stabilized in the region of 7·1. Addition of glucose to the suspending solution gave a more stable suspension over long periods, but was only satisfactory if care was taken in sterilization and if the glucose was of proved A.R. quality.

(4) Finally, the average day temperature in this climate varies between 82° and 85° F. with a relative humidity of 82–90 per cent., so that tissue autolysis is more rapid than it is under non-tropical conditions. Tissues in the short interval between removal and use were, therefore, kept on ice.

Reading results.

Incubation.—All tissue : : red cell suspension systems were incubated at 37° C. after setting up. Readings were in most cases taken at 12, 15, 18, 24 and 36 or 48 hours, but *always* at 18, 24 and 36 hours.

Estimation of lysis.—Tubes were first examined without shaking. Lysis usually exhibited itself as a clear zone of pigment in the supernatant fluid lying above the tissue : : red cell mixture, which collected in the bottom of the tube. Tubes were then vigorously shaken and samples of their contents were removed aseptically with Pasteur pipettes and examined microscopically.

The control red cell suspension was always sampled at the same time and used for purposes of rough comparison with the experimental tissue : : red cell suspension systems.

Time relations.—Only rough attempts were made to estimate the qualitative destruction of red cells over a period of time, but it was found that the degree of lysis bore a definite relation to the time during which the red cells and tissue had been in contact. Thus practically no visible lysis occurred in the first 12 hours of incubation, the red cell destruction being maximal between the 15th to 18th or 24th hours. After 24 hours as a rule, where lysis was still incomplete further destruction of cells occurred very slowly.

RESULTS.

The results of various experiments are given below for convenience in tabular form. All the systems referred to were set up in the manner described above.

Inhibition of lysis by heat.

The lytic action of all tissues examined was destroyed by heating the tissue at 80° C. before adding it to the cell suspension. The tissue was heated in suspending fluid after being cut to the requisite size for the experiment. The length of time of heating depended on the size of the tissue slice and was usually 5 minutes. Care was taken to heat the tissue long enough to affect all its substance in case any undamaged lytic agent was left in the central portions of the tissue.

DISCUSSION.

Nature of lytic agent.

It is considered that the lytic agent demonstrated in the above experiments is probably an enzyme—an erythrocytase. The probability of its being an enzyme is supported by the following observations :

(1) The time relations of the lysis of red cells by tissue are similar to those of an enzyme : : substrate reaction in that the process at first moves slowly, then gathers speed for a few hours and then slows again. More careful analysis of the time factors

TABLE I.—*The Lytic Power of Tissues.*

(a) *Tissue : : red cells of same species.*

Tissue examined.	Species. Guinea-pig.	Monkey. (*Cercopithecus.*)	Monkey. (*Erythrocebus patas.*)	Man.
Washed :				
Lung	L.	L.	L.	L.
Liver	L.	L.	L.	L.
Spleen	L.	L.	L.	L.
Kidney	L.	PL.	PL.	—
Heart muscle	L.	L.	—	—
Striped muscle :				
Psoas	—	PL.	PL.	—
Rectus	—	PL.	—	PL.
Marrow	—	L.	L.	—
Pancreas	—	PL.	—	—
Testes	—	L.	—	—
Lymph node	—	L.	—	PL.
Unwashed :				
All above tissues	0	0	0	0
Washings from vessels after exsanguination (monkey)	—	L.	—	—

Note.—L. = lysis ; PL. = partial lysis ; 0 = no lysis ; — = no experiment.

(b) *Tissue and red cells of different species.*

Tissue.	Cells. Human.	Guinea-pig.	Monkey.
Monkey :			
Kidney	0	0	L.
Liver	0	0	L.
Spleen	0	0	L.
Lung	0	0	L.
Guinea-pig :			
Lung	0	L.	0
Spleen	0	L.	0

Note.—L. = lysis ; 0 = no lysis.

involved in tissue lysis is required, but conditions in this station are not suitable for such work.

(2) The lytic agent is destroyed by heating the tissues to 80° C.

(3) The lysis is inhibited by minute traces of potassium cyanide and by low concentrations of mercuric chloride.

(4) The lytic agent has been found to be species specific and can be inhibited by titrable concentrations of serum.

It has not been possible to perform any respiratory experiments on the tissue : : red cell suspension systems, so that final proof of the enzymic nature of the lytic agent is still lacking.

TABLE II.—*Inhibition of Lysis by Various Factors.*

(a) *Using monkey red cell : : lung system.* (*Red cell : : spleen ; red cell : : kidney ; also tried.*)

Inhibitor.	Dilutions and results.						
i. Mercuric chloride	1/8000		1/16,000		1/32,000		1/64,000
Result	0		0		0		0
ii. Potassium cyanide	$1/2 \times 10^5$		$1/2 \times 10^6$		$1/2 \times 10^7$		$1/4 \times 10^7$
Result	0		0		0		L.
iii. Monkey serum	1/40		1/80		1/160		
Result	0		0		0		
iv. Human serum	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Result : Donor (i)	0	0	0	0	0	0	0
Donor (ii)	0	0	0	0	0	L.	L.

(b) *Using guinea-pig red cell : : tissue system.*

Inhibitor.	Dilutions and results.			
i. Potassium cyanide	$1/2 \times 10^5$	$1/2 \times 10^6$	$1/2 \times 10^7$	$1/4 \times 10^7$
Result	0	0	0	L.
ii. Guinea-pig serum	1/50	1/100	1/300	
Result	0	0	L.	
iii. Monkey serum	..	..	..	
Result	0	0	0	
iv. Human serum	1/10	1/50	1/100	1/250
Result : Donor (i)	L.*	0	0	0
Donor (ii)	0	0	0	L.

* Lysed cells in absence of tissue.

(c) *Controls.*

In all above experiments Part (a) and (b) :

Cells = 0.
Cells + tissue = L.

The time relations of the reaction, its inhibition by heat and potassium cyanide and its species specificity all indicate that the lysis is not merely a physical process brought about by some simple chemical substance or lysin.

The possibility that the lytic agent demonstrated by our experiments is only a product of the autolysis of dead tissue cannot be overlooked, but it is unlikely. First the tissues in the experiments were removed aseptically from anaesthetized living animals and were used within as short a time as possible (never more than two hours) after dissection. Secondly, the tissues were kept on ice in the period between excision and employment in experiments, thus reducing autolysis to a minimum. Thirdly, the tissue : : red cell system was found to be species specific. Again, the inhibition by dilutions of sera does not suggest the activity of autolytic products. The criticism that the tissues were in fact autolysing throughout the whole experiment cannot be answered, except by pointing out that such is the case in all tissue slice enzyme systems, and that we have found that tissue which has been incubated for 18 hours in contact

with red cells and which is then removed and placed in a fresh suspension of red cells produces lysis in the same time as in fresh tissue : : red cell systems. The presence of the lytic agent in the terminal washings from intact animals is perhaps the most conclusive evidence that it is *not* the produce of autolysis.

At this stage of our experiments the anatomical distribution of the lytic agent has not yet been worked out, apart from showing that it is present in most tissues. We do not know whether it is located in the cells or the tissue fluids, although its presence in the terminal washings from exsanguinated monkey tissue suggests the former.

The presence of the lytic agent in the terminal washings from exsanguinated monkeys (see Table I) further gives us an indication of its action in the intact animal, and refutes to some extent the criticism that our conclusions are based mainly on the results of experiments conducted without sections of tissue. It should be pointed out, in addition, that most respiratory and other experiments on tissue enzymes suffer from the same defect of being based on the activity of damaged tissue.

Nevertheless it seems highly probable that this lytic agent which can be demonstrated in tissues must be a factor in normal red cell lysis, and certainly in extravascular lysis occurring, for example, in such extravasations of blood as are found in bruising.

Our experiments give no indication of the actual site of such blood destruction in the intact body, although it appears likely that, at any rate in their passage through the finer capillaries, red cells must be in constant contact with the lytic agent.

The inhibitory factor.

It has been observed that unwashed fresh tissue will not lyse red cell suspensions, whereas the same tissue, if washed thoroughly in saline, will do so.

The few experiments carried out on the inhibitory effect of such tissue washings have given irregular results possibly because some of the lytic agent was removed at the same time, but in the case of both monkey kidney and monkey liver washings, inhibitory action can be demonstrated. It therefore appears that in the process of washing some diffusible inhibitory factor is removed from the tissue.

It is not clear whether this inhibitory substance is identical with that so easily demonstrated in circulating blood or, in fact, whether the inhibitory factor in unwashed tissue is any more than that already present in the contained blood, but it is possible to state that factors capable of inhibiting the activity of the tissue lytic agent occur both in the tissues themselves and in the blood serum.

The nature of this inhibitory factor (or factors) has not yet been worked out, but it is apparently diffusible, and the fact that it is non-species specific suggests that it will prove to be a relatively simple substance. It also appears to withstand drying, since reconstituted plasma has been shown to exert an inhibitory effect on lysis by tissue.

The control of lysis in the tissues.

We have thus demonstrated the presence of two opposing factors, a lytic agent present in the tissues and inhibitory factors (possibly a single factor) present in the tissues and in the blood serum. If this lytic agent is concerned in normal red cell destruction within the intact body, then it seems reasonable to infer that the degree of lysis occurring in the body at any given time must depend on the interaction of active lytic agent and inhibitor ; in other words, lysis depends on the balance set between the lytic agent on the one hand and the inhibitor on the other. Under normal conditions of metabolism, the balance is set at a point where destruction of red cells

continues at a basic level which runs parallel with red cell production. Alteration in the level of lysis would be governed mainly by quantitative or dynamic changes either in the lytic agent or inhibitor.

We therefore suggest that the investigation of the lytic agent content of the tissues and of the inhibitor content of sera and tissues in pathological states, such as blackwater fever, malarial and other lytic anaemias, etc., may give a clue to the processes underlying the increased lysis occurring in these conditions.

Our thanks are due to Dr. R. D. Reid, Senior Pathologist, Colonial Medical Service, for placing the facilities of his laboratory at our disposal, and to Brigadier R. A. Hepple for his help and interest during the course of these investigations.

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STAPHYLOCOCCAL ENTEROTOXIN—WITH SPECIAL REFERENCE TO THE KITTEN TEST.

F. FULTON.

From the Emergency Public Health Laboratory, Oxford, and the Sir William Dunn School of Pathology, Oxford.

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IN 1936 Dolman, Wilson and Cockcroft introduced the kitten test for the detection of enterotoxin in staphylococcal filtrates. Since then the test has been widely used in public health work and in the fundamental study of the nature of the enterotoxin. During 1941, staphylococci were isolated in this laboratory in large numbers from four foods concerned in four outbreaks of food poisoning. The history of these outbreaks was extremely suggestive of food poisoning of the toxin type, and yet three of the four strains gave negative kitten tests, and therefore could not be classified as enterotoxin producers.

We were thus led to a study of the validity of the kitten test.

I. *Technique of the kitten test.*

The strain was grown in Roux bottles for three days on a heart infusion medium containing 0·75 per cent. agar. Incubation was at 37° C. and the bottles were maintained in an atmosphere containing 10 per cent. CO_2. The agar was extracted by shaking with saline. This saline extract was then filtered through paper and centrifuged. The clear filtrate, adjusted to pH 7, was boiled for 20 minutes, and 2 ml. were injected intraperitoneally into a ½ kg. kitten. Under these conditions the strain was deemed to have produced an enterotoxin if the kitten vomited within about two hours.

II. *The value of the kitten test.*

(i) A strain of staphylococcus (Jordan 7) was grown on soft agar in 10 per cent. CO_2.

A saline extract of this culture was centrifuged, and volunteer A drank 5 ml. in a glass of water. Vomiting began two hours later and continued for eight hours. And yet this same extract, when boiled for 20 minutes, and injected intraperitoneally into a kitten, produced no symptoms.

(ii) Another strain of staphylococcus (C13344) was grown in a similar manner. Volunteer A drank the extract, but neither 5 ml. nor 10 ml. provoked any symptoms. This same extract, after boiling for 20 minutes, provoked severe vomiting in a kitten after intraperitoneal injection. Many subsequent tests have confirmed the toxicity of this strain for kittens. It appeared, therefore, that the factor in staphylococcal filtrates which was causing the kittens to vomit in the kitten test was not identical with the factor which was causing the volunteer to vomit.

III. *Experiments to determine the factor in staphylococcal filtrates which causes vomiting in kittens.*

1. Of the four strains of *Staphylococcus aureus* isolated from the food-poisoning outbreaks, the three kitten-negative strains produced only alpha lysins, whereas the kitten-positive strain produced in addition a potent beta lysin.

(*a*) If an unheated extract of the Wood 46 strain, which contains alpha but no beta lysin, is injected intraperitoneally into a kitten, the animal vomits within about ten minutes, there is extreme collapse and it dies overnight. At post-mortem there is obvious peritonitis. If the extract is boiled for 20 minutes before injection the kitten does not vomit. This is true of all the pure alpha-producing strains which we have tested. The initial lysin titres of such strains are between 1/100 and 1/1000; after boiling for 20 minutes the titre is less than 1/20 (Table I).

TABLE I.—*The Relation Between the Haemolysins and the Kitten Test.*

	Unheated.			Boiled for 20 minutes at pH 7.		
	Titre.		Kitten test.	Titre.		Kitten test.
	α.	β.		α.	β.	
			2 *ml. i.p.*			4 *ml. i.p.*
Wood 46	1/1280	0	Vomited after 5, 15, 20 minutes	1/10	0	No effect.
			1 *ml. i.p.*			2 *ml. i.p.*
C13344	1/2560	1/5000	Vomited after 10, 15, 30 minutes	1/16	1/256	Vomited after 25, 45 minutes.

i.p. = intraperitoneally.

(*b*) If an unheated extract of a strain which produces an alpha lysin and a potent beta lysin is injected intraperitoneally into a kitten, the animal vomits within about ten minutes, there is extreme collapse and it dies overnight. At post-mortem there is peritonitis. If the extract is boiled for 20 minutes before injection the kitten vomits within about half-an-hour, shows little collapse and may or may not die overnight. At post-mortem there is little evidence of peritonitis. The boiling has reduced the alpha lysin titre to less than 1/20, and the beta lysin titre from about 1/5000 to between 1/300 and 1/100 (Table I). If the initial beta lysin titre is low, the boiling destroys the lysin and the strain is kitten-negative after boiling.

It seemed that a possible explanation of these observations was that both the alpha

and the beta lysins, in sufficient amount, were capable of causing kittens to vomit when injected intraperitoneally. In order to test this hypothesis it was necessary to purify the lysins.

2. *Purification of the lysins.*

(*a*) Purification of the alpha lysin of the staphylococcus Wood 46.

Several methods of purification have been described. After preliminary precipitation with two and a half volumes of acetone, at pH 4 in the cold, the method of Boivin and Izard (1937) was used. The acetone precipitate was redissolved in saline and precipitated in the cold with trichloracetic acid, by adjusting the reaction to pH 3·5. The precipitate was centrifuged down and dissolved in N/10 sodium carbonate, which was then neutralized.

A batch of the purified material with an alpha lysin titre of 1/80 had a dry weight of 0·6 mgm./ml., and a nitrogen content of 0·068 mgm./ml.

(*b*) Purification of the beta lysin of the staphylococcus C13344.

(i) Preliminary extraction : The strain was grown on a soft agar medium for three days in an atmosphere of 90 per cent. air and 10 per cent. CO. Fifty ml. of the agar culture was extracted with 20 ml. of isotonic saline.

Extract :

Alpha titre	1/640.
Beta titre	1/5000.

(ii) Precipitation with acetone : Precipitation of the lysins by acetone was studied over a pH range of 3 to 9. It was found that the beta lysin was optimally precipitated at pH 9, whereas the optimal pH for the alpha lysin precipitation was 4.

Twenty ml. of M/15 borate buffer of pH 9 were added to each 50 ml. of the soft agar culture. The agar was broken up by shaking for a few minutes, and the agar was removed by filtration through paper. The filtrate was centrifuged and the clear supernatant fluid was passed through a Seitz EK filter. This filtrate was cooled to 0° C., and one and a half volumes of cooled acetone were added. A gelatinous precipitate formed which was centrifuged down with precautions taken to prevent the temperature rising above + 2° C.

The precipitate was re-dissolved in M/15 phosphate buffer of pH 6, using an amount 1/3 of the initial volume.

Acetone precipitate :

Alpha titre	1/128.
Beta titre	1/10,000.

(iii) Adsorption and elution from aluminium hydroxide : The beta lysin was found to be easily adsorbed on to a number of adsorbents, but aluminium hydroxide was chosen because of the marked effect that the pH had upon the amount adsorbed. Thus, at pH 6 adsorption was very good, whereas at pH 9 very little was adsorbed. Since the optimal pH for the alpha lysin adsorption was about 4, and since the alpha lysin was very unstable at alkaline pH, it was possible to adsorb the beta lysin at pH 6 and elute at pH 9, and thus secure a very good separation of the two lysins.

The acetone precipitate in the phosphate buffer at pH 6 was adsorbed with aluminum hydroxide of the C gamma variety. The adsorbent was washed once with an equal volume of the phosphate buffer, and the beta lysin was then eluted into an equal volume of M/15 borate buffer of pH 9. The elution was effected on a gentle

shaking machine at 37° C., the optimal time varying between 5 and 20 minutes according to the batch of adsorbent.

Eluted lysin :

Alpha titre	<1/2.
Beta titre	1/2000.

(iv) Estimate of the degree of purification achieved : The purified beta lysin in the borate buffer was water-clear and colourless. It was very stable if kept in the ice-chest.

	Titre.	Dry weight, mgm./ml.	Nitrogen, mgm./ml.
Extract	1/5000	12·7	2·6
Acetone precipitate	1/10,000	8·8	0·198
Eluted lysin	1/2000	0·2	0·025

It was unlikely that the purification had resulted in a homogeneous preparation, and so an attempt was made to detect other enzymes in the purified preparation. No splitting of fats, phosphatides, nucleotides, phosphoric esters or carbohydrates could be detected, using egg yolk and sheep red cell stromata as substrates. There was, however, a proteolytic enzyme which split both egg albumin and casein. With casein an opacity developed and there was a rise in the acid-soluble phosphorus as well as in the non-protein nitrogen. The proteolytic enzyme had properties which differed from those of the beta lysin. Thus, in an experiment in which the purified preparation was heated at various pH for half an hour at 56° C., it was found that the beta lysin was least stable at pH 7, whereas the proteolytic enzyme was most stable at pH 7. Thus it is presumed that the beta lysin and the proteolytic enzyme are not identical. The beta lysin was also found to produce an opacity in some human sera, exactly simulating the Nagler reaction. The factor producing this opacity seemed to be identical with the beta lysin. There was no egg yolk reaction and no lecithinase.

3. *Test of the purified lysins in kittens.*

(*a*) The purified alpha lysin caused vomiting when injected intraperitoneally into kittens. Two ml. of a preparation with a titre of 1/100 gave uniformly positive results, but less lysin than this did not always provoke vomiting. Large amounts killed the animal and at post-mortem there was evidence of peritonitis. The toxicity was destroyed by neutralizing the alpha lysin with a specific anti-serum.

(*b*) The purified beta lysin caused vomiting when injected intraperitoneally into kittens. The animal was extremely sensitive to this lysin. Thus two gamma injected intraperitoneally caused vomiting, four gamma subcutaneously gave rise to gelatinous oedema and haemorrhagic necrosis, and 200 gamma intravenously killed a 0·5 kg. cat in three hours. Using minimal doses to provoke vomiting no peritonitis was induced, but with larger amounts there was peritonitis. The toxicity was destroyed by neutralizing the beta lysin with a specific anti-serum.

It is concluded, therefore, that both the alpha and the beta lysins, or toxins closely associated with them, are capable of giving rise to vomiting in kittens when injected intraperitoneally. Under the conditions of Dolman's test, the beta lysin is the only one which escapes destruction by boiling in sufficient amount to be toxic. Thus the beta lysin is the responsible factor in the test.

Since completing this work, a study of the literature has shown that Kojima and Kodama (1939) reached a similar conclusion, and Slanetz (1941) has published a note to the same effect.

It seems probable that the vomiting is induced by a local irritation. Injection intraperitoneally into kittens of many substances causes the kitten to vomit. Among these are croton oil, mustard oil, suspensions of meat and fish foods, strong brine and evaporated milk.

IV. *Experiments to determine the factor in staphylococcal filtrates which caused the volunteer to vomit.*

All the filtrates were tested by volunteer A, who had been proved susceptible to staphylococcal enterotoxin. The strains were grown on soft agar in 10 per cent. CO_2. The saline extracts were centrifuged clear and were drunk in a glass of water on an empty stomach.

(i) *Filtrates of the Wood* 46 *Strain.*—This strain produced an alpha lysin with a titre of 1/640, but no beta lysin. The filtrate was not toxic by mouth in a dose of 10 ml. The alpha lysin purified from this filtrate was also not toxic by mouth.

(ii) *Filtrates of the C*13344 *strain.*—This strain produced an alpha lysin with a titre of 1/640, and a beta lysin with a titre of 1/5000. The filtrate was not toxic by mouth in a dose of 10 ml. The beta lysin purified from this filtrate was also not toxic by mouth.

(iii) *Filtrates of the Jordan* 7 *strain.*—This strain produced an alpha lysin with a titre of 1/160, and a beta lysin with a titre of 1/32. The filtrate was passed through a Seitz EK filter, and it was found that a dose of 1 ml. produced a reaction of moderate severity. Vomiting began in two hours, but after three hours recovery was rapid, being almost complete in six hours.

(*a*) Toxicity by mouth of ether extracts of the enterotoxic filtrate (Jordan 7) :

It was claimed by Jordan and Burrows (1933), using monkey feeding as the biological test method, that the enterotoxin could be completely extracted from acid aqueous solution by ethyl ether or chloroform. Accordingly a filtrate, proved toxic in a dose of 1 ml., was brought to pH 2 and extracted with ether. The ethereal layer was pipetted off and the aqueous layer discarded. The ether was removed *in vacuo*, there being nothing but a little fat apparent in the residue. An amount of this residue, suspended in water, was drunk, corresponding to 1 ml. of the filtrate. It was not toxic.

(*b*) Toxicity by mouth of purified beta lysin of the enterotoxic filtrate (Jordan 7) :

An attempt was made to purify the beta lysin, using a batch of filtrate proved toxic in a dose of 1 ml. Owing to the very low initial beta lysin titre (1/32), it did not prove possible to continue the purification beyond the acetone stage, since the losses were too great during the adsorption and elution from aluminum hydroxide.

An amount of the acetone fraction was taken (4 ml.), such that the beta lysin content was equal to that contained in 1 ml. of the crude filtrate. As precipitation with acetone at pH 9 was very unfavourable to the alpha lysin, the amount of alpha lysin taken in this 4 ml. was only 1/8 of that contained in 1 ml. of the crude filtrate.

The acetone fraction in this dose was not toxic by mouth.

(*c*) Toxicity by mouth of purified alpha lysin of the enterotoxic filtrate (Jordan 7) :

A Seitz filtered extract was buffered at pH 4 and precipitated with two and a half volumes of acetone. The precipitate was dissolved in half a volume of saline. The alpha lysin was again precipitated by adding N/2 trichloracetic acid until a pH of 3·5 was reached. The precipitate was centrifuged down in the cold, re-dissolved in N/10 sodium carbonate and then adjusted to pH 7. The final alpha lysin titre was 1/40, the initial alpha titre of the crude filtrate was 1/160. There was no demonstrable beta lysin in the purified product.

Two ml. of this purified alpha lysin were taken. In two hours there was nausea and one bout of vomiting, but the effect was less than with 1 ml. of the crude filtrate. Next day 4 ml. were taken, this having an amount of alpha lysin corresponding to 1 ml. of the crude filtrate. This dose gave a reaction of the usual severity.

These experiments have been too few to permit of any dogmatic conclusion, but it seems that a higher degree of purification must be achieved before it is possible to differentiate the enterotoxin from the alpha lysin of this strain.

DISCUSSION.

Since the kitten test is not detecting the same factor as that which causes food poisoning in man, much of the published work on the staphylococcal enterotoxin must be reconsidered.

Using the valid test method of the susceptible human volunteer, there is conflicting evidence on several fundamental points. Thus Jordan (1930) concluded that most strains of *Staphylococcus aureus* were capable of enterotoxin production, whereas Dolman (1934) concluded that this property was confined to a few strains of staphylococci. What does seem clear is that many strains rapidly lose their enterotoxic capacity on subculture. Nor has it been conclusively proved that the enterotoxin is distinct from the alpha lysin of the food-poisoning strains (Jordan, 1930 ; Dolman, 1934). That not all alpha lysins, however, are toxic by mouth has been shown in the present work.

Much stress has been laid on the supposed difference between the heat stability of the alpha lysin and the enterotoxin, the enterotoxin being said to resist boiling, whereas the alpha lysin was easily destroyed at 60° C.

Jordan (1930) showed that the enterotoxin was destroyed or very much weakened by heating at 60° C. for 30 minutes. Later (Jordan *et al.*, 1931) he found that it often resisted 100° C. for 30 minutes. The alpha lysin behaves in the same way.

Landsteiner and von Rauchenbichler (1909) recorded the fact that the inactivation of staphylococcal haemolysin was easily achieved by heating at 65° C., but that, paradoxically, the destruction was much less complete at temperatures of 80° and 100° C. This is supported by Kodama and Nisiyama (1938) and by Beumer (1939). The instability at 65° C. is also shown by crude beta lysin (Kodama and Kojima, 1939).

We have confirmed these facts, and we have found that the instability is less marked with the purified than with the crude beta lysin. We have also found that the proteolytic enzyme in the purified beta lysin preparation, and the alpha lysin and the lecithinase of *Cl. welchii* culture filtrates, all show the same instability at 65° C., so that the phenomenon would seem to be of general importance.

On the evidence at present available, it is thus impossible to say whether the enterotoxin is distinct from or identical with the alpha lysin of the food-poisoning strains.

CONCLUSION.

In any future investigation of the enterotoxic problem it is of great importance that valid test methods should be employed.

Hammon (1941) has introduced a method which consists in the intravenous injection of heated extracts into cats. He claims that enterotoxic extracts provoke vomiting, but the method has not been tested in parallel with a valid feeding test. Great caution should be exercised in the interpretation of vomiting after intravenous injection (Woolpert and Dack, 1933). In the present work it was found that the purified

alpha lysin usually caused vomiting when injected in sufficient amount intravenously into kittens. Sometimes only diarrhoea was provoked. The animals died overnight. If large amounts of purified beta lysin of the order of 200 gamma were injected intravenously, vomiting was provoked and the kittens died within a few hours. With smaller doses of the order of 50 gamma, the kittens usually suffered from diarrhoea within half an hour of injection, but they did not vomit.

Hammon claims that all the alpha and beta lysins are precipitated from the crude extract by 75 per cent. saturation with ammonium sulphate, and that the enterotoxin is left behind in solution. However, it does not seem that 75 per cent. saturation can be relied upon to precipitate all of the lysins—though it may occasionally do so.

At present the biological test methods most reliable for the detection of enterotoxin are feeding methods. By far the most satisfactory method is a properly controlled human volunteer method. The volunteers must be proved to be susceptible, and they must be used for only a few weeks, as immunity seems to be attained fairly rapidly. However, if any extensive chemical purification is to be attempted, such a test method is rendered nearly impracticable, and it will be necessary to fall back upon other methods.

The feeding of rabbits and guinea-pigs does not promise to be of any value, as the claims of Borthwick (1933) have not been substantiated. The monkey feeding test has been widely used. Those who have had most experience of it seem to consider that the response is variable, but it would be valuable to reinvestigate this method, particularly with reference to the species of monkey most suitable, and the conditions under which reliable results may be obtained.

Cats are not affected by the oral administration of small doses (5 ml.) of soft agar extracts which are toxic in 1/5 of that amount to man. But Dolman (1936) claims that if 50 ml. amounts are fed, the cats suffer from projectile vomiting after about two and a half hours. It would seem to be worth while to investigate this method, since it would be simple to concentrate the extracts *in vacuo*.

Jones and Lochhead (1939) grew the strains on a special starch medium, and claim to have produced vomiting in cats by feeding only 5 ml. of the extract.

SUMMARY.

1. The intraperitoneal kitten test for the detection of enterotoxic strains of *Staph. aureus* has been investigated:

(i) A kitten-positive extract was non-toxic by mouth to a susceptible human volunteer, and a kitten-negative extract was toxic to this volunteer.

(ii) Many other substances injected into the peritoneal cavity of kittens induced vomiting. The test is not specific for staphylococcal enterotoxin, but is rather an index of peritoneal irritation.

It is concluded that the intraperitoneal kitten test cannot be relied upon to detect either the presence or the absence of staphylococcal enterotoxin.

2. The responsible factor in the kitten test:

(i) It was found that the kitten-positive strains produced potent beta lysins, whereas the negative strains did not.

(ii) Purified alpha and beta lysins injected intraperitoneally into kittens induced vomiting.

(iii) Boiling for 20 minutes extracts which contained alpha and beta lysins destroyed most of the alpha lysin leaving insufficient to induce vomiting, but there was enough beta lysin to cause the kitten to vomit.

It is concluded that the beta lysin, or some toxin closely associated with it, is the responsible factor in the kitten test.

3. A method for the purification of the beta lysin is described. The purified beta lysin fraction contained a proteolytic enzyme, which was probably a distinct enzyme not related to the beta lysin.

4. Human feeding experiments with a susceptible human volunteer have shown that the beta lysin investigated was not toxic by mouth, and that some extracts containing alpha lysins were also not toxic by mouth. On the other hand, in a toxic extract it proved impossible to separate the enterotoxin from the alpha lysin of that extract.

5. The evidence on which the distinction between the enterotoxin and the alpha lysin has been based is inconclusive. The supposed heat stability of the enterotoxin as against the heat lability of the alpha lysin can no longer be maintained, since it has been found that the destruction of the alpha lysin at low temperatures is apparent, not real, and that in fact less inactivation of the alpha lysin occurs by boiling for a given time than by heat at 65° C.

6. It is suggested that the problem of the food-poisoning staphylococci should be reinvestigated, using valid test methods, of which at present the human volunteer is the only one on which reliance can be placed.

It is a pleasure to record my debt to Dr. E. Chain for much help in the biochemical part of this work.

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A STUDY OF THE MORPHOLOGY AND DEVELOPMENT OF THE MEGAKARYOCYTES.

J. JAPA.

From the Polish Medical School, and the Institute of Animal Genetics, University of Edinburgh.

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MEGAKARYOCYTES, first so called by Howell (1890), represent the most striking and least known elements in the red bone marrow. Their origin, development and structure, and even their function are still under discussion, although at present an almost generally accepted opinion prevails, attributing to them the platelet-producing activity.

The present study concerns two closely connected problems: (A) The morphology and structure of the nuclear body, and (B) the mode of development of the megakaryocytes. The opinions of the many authors investigating the development of these cells differ considerably. It is variously assumed that they arise—

(1) From uninuclear cells by repeated "amitotic" division or fragmentation of the nucleus without the division of the cytoplasm (Arnold, 1883; Jordan, 1918).

(2) By irregular or multipolar division (Kostanecki, 1892; Heidenhain, 1894; Foà, 1899; Levy, 1921; Maximow, 1927; Downey, Palmer and Powell, 1930; Bunting, 1932; Potter and Ward, 1940).

(3) From uninuclear cells by overgrowth of the nucleus and cytoplasm (Howell, 1890; Klaschen, 1922; Kaufman, 1929; Rothemel, 1930).

(4) Through the fusion of several uninuclear cells (Bianchi, 1921; di Gugliemo, 1925; Baserga, 1932). Detailed reviews of the literature concerning these views may be found in the works of Jordan, Kaufman, Rothermel, and Potter and Ward cited above.

MATERIAL AND TECHNIQUE.

The ten samples of normal red marrow investigated were taken from ten male patients of the Royal Infirmary,* Edinburgh, aged between 20–40. The cytological examination of the peripheral blood and marrow films showed no signs of any affection of the hemopoietic system. In addition one sample from a case of untreated pernicious anaemia (marked P.A. in tables) was examined (a woman, aged 60: Hb 36 per cent. [Sahli], red cells 1·5 million, colour index 1·2, white cells 4000, reticulocytes 1 per cent.), and one from a case of thrombopenic purpura with splenomegaly (marked Pra in tables: a man, aged 45, slight anaemia, platelets about 30,000 per c.mm.).

Marrow samples were taken from the sternum by aspiration. Particles were fixed and stained in aceto-carmine, and prepared by the squash method as described in a previous paper (Japa, 1942). To ensure satisfactory results three points ought to be emphasized: a thick enough needle for sternal puncture, the use of isolated particles for preparations, and good dispersion of the disintegrated tissue.

* The author wishes to express his sincere thanks to Dr. T. R. R. Todd for permission to obtain the material for this study.

RESULTS.

A. *Morphology of the Megakaryocytes.*

Megakaryocytes fixed and stained in this way are much larger and better preserved than those subjected to any other method of preparation. They exhibit a great range

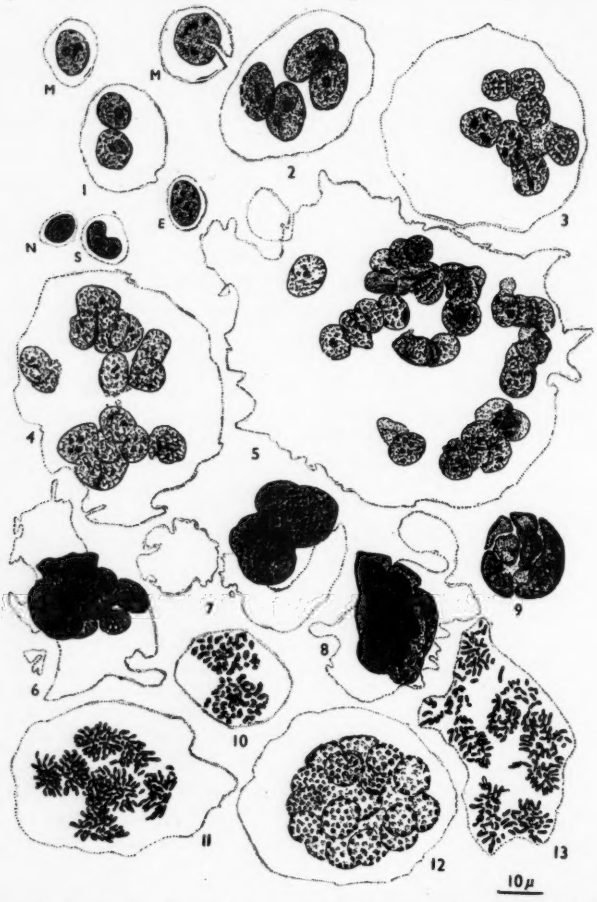

FIG. 1 (Semi-schematic drawings).—M, myelocytes; E, early erythroblast; N, late erythroblast; S, staff cell. 1, 2, 3, 4, 5, Megakaryocytes with 2, 4, 8, 16 and 32 nuclei respectively. 6, 7, 8 and 9, Megakaryocytes in various stages of the degeneration. 10, Prophase in a cell with two nuclei. 11, Four anaphase figures in a megakaryocyte. 12, Prophase or late telophase in a megakaryocyte (16 nuclei probably). 13, Multiple metaphase figures in a megakaryocyte.

of variation in size, the smallest being some 30μ in diameter and the largest up to 100μ. Their outline is usually round or oval, but occasionally it is very irregular. The cytoplasm stains very lightly and its structure is homogeneous, though often it shows less staining round the nuclear body. The amount of the cytoplasm seems to be more or less proportional to the size of the nuclear body.

The nuclear body in megakaryocytes is composed of small nuclei in definite numbers.

In some cells they are well isolated but more often they stick together, imitating one giant, lobulated nucleus or forming an extremely polymorphous body cut by the more or less distinct boundaries of the individual, component nuclei. The size of the individual nuclei is approximately the same as in erythroblasts, i.e. it varies between 6–10μ. They are apparently larger in cells with few nuclei and their size seems to decrease with the increase in number in a cell. The individual nuclei within a cell are very similar in size and in morphology ; the resting nuclei show a uniform, reticular structure, a little less dense than that of erythroblasts, and often contain irregular patches of darker chromatin. Sometimes they show protrusions of chromatin running into other nuclei. There was no distinct evidence of nucleoli within the nuclei.

The counting of the nuclei in well prepared squashes is not difficult, and it reveals that the megakaryocytes fall into classes with gradually increasing numbers of nuclei.

Among the nucleated cells in marrow a small proportion, 25–30μ in diameter, i.e. the size of large myelocytes, contain two nuclei. The nuclei measure about 7–9μ, and they stain more deeply than those of other types described below (Fig. 1, 1).

Cells with four nuclei are far more numerous. They vary from about 30 to 40μ, and are round in shape with distinct cytoplasmic outlines. The nuclei lie in one or two groups, or in some cells are well isolated ; they are a little larger than in binucleated cells, about 10μ, and the structure of their chromatin is less dense (Fig. 1, 2).

The most common among the multinucleated cells in normal marrow are those of up to 60μ, with a large disc of cytoplasm, and a polymorphous nuclear body with distinguishable areas of individual nuclei. In some the nuclei are lying in two groups, while in others all of them are well separated ; the number in one cell is eight, and they vary from 7–9μ (Fig. 1, 3).

Less common are the still larger cells, up to 90μ, with enormously developed cytoplasm, and a huge extremely polymorphous nuclear body, about twice as large as in the previous type, composed of 16 nuclei. The nuclei may form one ball or ring-shaped structure, or sometimes they are in two or more groups, but separate nuclei can be easily distinguished ; 7–8μ is their diameter (Fig. 1, 4).

Very rarely one can see even larger cells up to 100μ with an enormous nuclear body, containing most probably 32 nuclei, forming most bizarre shapes. The diameter of the individual nuclei is about 6–7 μ (Fig. 1, 5). No cells were found with more nuclei than that.

Besides the " normal " megakaryocytes a certain proportion of so-called " degenerating " or " involuting " forms are to be seen in the preparations. The first signs of degeneration seem to be the condensation and the more intensive staining of the chromatin, beginning on the inner side of the nuclear membrane ; at a later stage constrictions develop in the nuclei, resulting in a still more polymorphous appearance of the nuclear body. Sometimes the boundaries of the individual nuclei are no longer distinguishable, giving the appearance of a single giant nucleus (Fig. 1, 6, 7, 8). At the same time the outline of the cell becomes irregular, the cytoplasm breaks into particles and later disappears altogether, so that in the last stage only naked, lobulated or ball-shaped, dark stained pycnotic nuclear bodies are to be seen (Fig. 1, 9). It seems that the degeneration starts when the cell has 8 nuclei, sometimes later, in cells with 16 or 32 nuclei ; rather exceptionally in normal marrow the cells with 4 nuclei may show signs of degeneration.

The proportion of megakaryocytes with 2, 4, 8, 16 and 32 nuclei was very constant in all samples of normal marrow examined (Table I). Averaging the counts for the ten normal cases the respective percentages are : 2·5, 25·5, 53, 18 and about 1. The proportion was calculated without including degenerating forms.

TABLE I.—*Proportion of Megakaryocytes with Different Numbers of Nuclei.*

Sample:	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	Total number.	Average. (per cent.).
Population . .	215	197	157	124	197	149	80	121	155	190	1585	..
Percentage with												
2 nuclei . .	4·2	2·7	1·3	1·5	3·6	3·3	2·5	1·7	1·9	1·7	40	2·5 (± 1)
4 „ . .	24·6	21·9	23·5	30	30·4	25·5	22·5	19	27·1	28·4	405	25·5 (±3·2)
8 „ . .	52·1	50	54·8	47	53·4	53·7	58·6	56·3	53·5	50·5	834	53 (± 3·2)
16 „ . .	18·1	23·4	19·2	20	12·6	17	16·4	20·6	17·5	18·4	290	18 (±2·7)
32 „ . .	1	2	1·2	1·5	..	0·5	..	2·4	..	1	16	1 (± 0·6)

The proportion of degenerating megakaryocytes was also constant in all the samples of normal marrow, being about 36 per cent. of the total number of megakaryocytes counted (Table II).

TABLE II.—*Proportion of Degenerating Cells.*

Sample:	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	Total.	Average.
Population . .	307	289	250	183	320	205	130	200	270	313	2467	..
Percentage degenerating .	29·9	31·8	37·2	32·2	38·4	27·3	38·4	39·5	42·6	39·2	..	35·7 (±5)

The size of the different classes of megakaryocytes and the proportion of degenerating cells in the two samples of pathological marrow examined show considerable differences in comparison with normal red marrow (Table III). In the case of pernicious anaemia the class of cells with 4 nuclei was the largest, the number of those with 8, and especially with 16 nuclei was much decreased. The percentage of degenerating cells was similar to that in normal marrow, but most of them belonged to the group with 4 nuclei, not that with 8. In the case of thrombopenic purpura a greatly increased proportion of cells with 16 and 32 nuclei was found, while the proportion of degenerating forms was much diminished (graphic illustration Fig. 2).

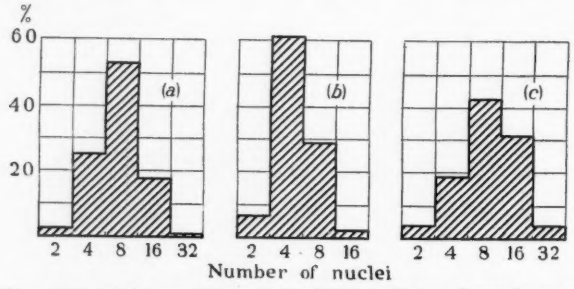

FIG. 2.—Graphic illustration of the proportion of the different classes of megakaryocytes according to number of nuclei. (*a*) Average of the ten samples of normal marrow examined; (*b*) in the case of pernicious anaemia; (*c*) in the case of thrombopenic purpura.

TABLE III.—*Percentage of Different Types of Megakaryocytes in Two Pathological Samples of Marrow.*

Class:	2.	4.	8.	16.	32.	Degenerating.	Population.
P.A. . .	6·9	61·1	29·2	2·8	..	43	252
Pra . .	3·9	19·2	42·6	30·7	3·6	13	726
10 normal cases .	2·5	25·5	53	18	1	35·7	2467

B. *Development of the Megakaryocytes. Mitosis in Multinucleated Cells.*

When examining the megakaryocytes in samples of bone marrow one comes across mitotic figures, and on looking through fairly extensive numbers it appears that these figures are by no means exceptional; on the contrary, the process seems to be the regular phenomenon among the giant cells in red marrow. Counts showed that the proportion in mitosis was similar in the 12 samples examined and averaged 17 per 1000 cells (Table IV).

TABLE IV.—*Proportion of Megakaryocytes in Mitosis.*

Sample:	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	P.A.	Pra.	Total.
Number of cells.	400	650	600	183	500	400	400	270	1000	313	1000	726	6439
Number of mitoses	8	12	10	2	6	7	6	4	12	8	15	20	110
Pro mille	20	18·4	16·6	11	12	17·5	15	14·8	12	25·5	15	26	17 (± 5)

A study of mitotic processes in the megakaryocytes reveals some very interesting features. The most important characteristic is that each of the nuclei composing the nuclear body of a megakaryocyte divides independently, forming its own spindle and typical mitotic stages. The second is that all nuclei composing the nuclear body undergo division synchronously (Fig. 1, 10 to 13). Examination of the mitotic figures proves that the division is regular; each of the component nuclei forms a normal bipolar spindle, and in each dividing nucleus typical mitotic stages, pro-, meta-, ana-, and telophase, could be observed. During the metaphase the chromosome complexes of each nucleus form independent equatorial plates. The axes of the various spindles have no relationship to each other. The chromosomes could not be counted exactly, but comparing them with those of other marrow cells of the same size it seems most probable that they represent the diploid number (48). The size of the equatorial plates is the same for each nucleus in the same cell. The large rod- and V-shaped chromosomes occupy the periphery of the equatorial plate; the smaller chromosomes are grouped in the centre. The sister chromatids of some chromosomes could clearly be seen. In the anaphase stage chromosome lagging was not observed. The chromosomes of one complex do not mix with those of others, which indicates that the anaphase spindles of the separate nuclei are independent. The end-result of the division is the formation of two daughter-nuclei of similar size. There does not seem to be any actual "fusion" between the nuclei; they may aggregate into groups, but it is very probable that they still retain their own nuclear membrane. Division of the cytoplasm, either simultaneously with the division of the nuclei or later, was never observed.

The proportion of the four mitotic stages in megakaryocytes, calculated from 110 observed instances of division (Table V), was similar to that found among other nucleated marrow cells taken together (Japa, 1942). The largest absolute number of

TABLE V.—*Proportion of the Different Mitotic Stages in Megakaryocytes.*

Sample:	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	P.A.	Pra.	Total.	%.
Prophase	5	3	4	1	3	3	4	3	8	3	7	9	53	48
Meta- and anaphase	2	9	6	1	3	4	1	..	3	4	8	9	50	46
Telophase	1	..	..	..	..	..	1	1	1	1	..	2	7	6

mitoses was found among the cells with 4 and 8 nuclei. When the proportion is calculated in relation to the number of cells in the respective classes, however, they are found to be most frequent in the type with 2 nuclei, less frequent in those with 4 and 8 nuclei, and very rare in cells with 16 nuclei; no example was found in cells with 32 nuclei.

c. *Proportion of Megakaryocytes to Other Marrow Cells.*

The proportion of megakaryocytes to the other nucleated marrow cells was also estimated. The technique of the counting was as follows: Preparations were used in which the distribution of cells was more or less uniform. In each sample 100 megakaryocytes were counted, including degenerating forms, and all other nucleated cells in every tenth field. In this way the approximate number of marrow cells per 100 megakaryocytes was calculated for each case examined. In the ten normals the

respective figures in thousands were : 60, 25, 55, 35, 20, 30, 35, 55, 40, 55, averaging 41 (± 13) thousand per 100 megakaryocytes. Both pathological cases gave a count of 50 thousand.

DISCUSSION.

The presence of giant cells with many nuclei has been observed in red bone marrow by many investigators. Howell (1890) distinguished two types of giant cells, " polykaryocytes "—multinucleated cells, and " megakaryocytes "—giant cells with apparently one giant nucleus. Arnold (1883), Jordan (1921), and others observed transitional forms between both types. Jordan believed that polykaryocytes develop from young megakaryocytes by constrictions, lobulations, and fragmentation of the nucleus. Rosenthal (1938) found that both types show the same morphological structure and both have the same platelet-producing activity. Levy (1921) maintained that megakaryocytes are primarily multinucleated, and later the separate nuclei fuse together in one " synkaryon." Bunting (1932) accepts multinucleated structure of the nuclear body, and so do Whitby and Britton (1942) in their recent text-book of blood diseases. On the contrary Klaschen (1922), Kaufman (1929) and Rothermel (1930) state that the nucleus of the megakaryocyte is probably always single.

The main cause of these contradictory statements seems to be the inadequate technique of marrow preparations used by most authors. The commonly used smear films and panchromatic staining give pictures which are hardly suitable for the study and sound interpretation of the structure of the nuclei, especially in megakaryocytes. Fixing and staining in aceto-carmine has the advantage that the structure is very well preserved, and the squash method of preparation makes possible the isolation of the nuclei composing the nuclear body. The examination of megakaryocytes in such preparations reveals that the nuclear body is always composed of a definite number of individual nuclei. In view of this fact the name " polykaryocyte " would be more appropriate for the giant cell in red marrow than the now generally used " megakaryocyte."

The second point resulting from the present study is that the nuclei of megakaryocytes undergo regular mitotic division. The process is mentioned by almost every investigator of these cells ; some believe that mitosis is extremely rare and rather exceptional, or actually pathological, while others accept mitotic division as the means of development of the giant cells. Some authors give good pictures of mitosis in megakaryocytes, but their interpretation is not correct, for they assume that the figures represent division by multipolar spindle (van Bambeke and van der Stricht, 1890 ; Kostanecki, 1892 ; Levy, 1921 ; Maximow, 1927 ; Downey *et al.*, 1930). Bunting (1932) and others compared these figures to those observed in malignant tumour cells. Potter and Ward (1940) believe that the mitosis has the object of multiplication of the chromosomes, and that the result of such division is the formation of a cell with one polyploid nucleus.

The examination of the mitotic figures in aceto-carmine preparations shows that mitosis in megakaryocytes is normal, i.e. all nuclei in a cell divide synchronously but independently, each of them by separate bipolar spindle. No evidence of abnormal mitosis or division by multipolar spindle was observed. In the metaphase the chromosomes are not scattered indiscriminately in one plate, but each nucleus forms its own equatorial plate ; in the anaphase there was no evidence of non-disjunction, or lagging of chromosomes, or of chromosome bridges as described in aceto-carmine preparations of malignant tumour cells by Koller (1942). All typical stages could be observed, including the telophase and the formation of two daughter-nuclei. The most important proof of the regularity of mitosis in megakaryocytes is the fact that the number of nuclei present is regularly of the 2^n order, i.e. 2, 4, 8, 16 or 32 nuclei. The only possible

explanation of that is the regular, independent mitosis which occurs synchronously in all the nuclei in the cell and which is not followed by division of the cytoplasm.

Most investigators agree that the cytoplasm does not participate in division. Van Bambeke and van der Stricht (1891) assume the possibility of the division of the cytoplasm long after the completion of the mitosis in nuclear body, creating two daughter giant cells. In aceto-carmine preparations no example of the division of cytoplasm was observed, although the occurrence of groups of two or more giant cells close together makes the idea suggestive.

The observations described above lead to the conclusion that the megakaryocytes develop from some uninuclear cells by division of the nucleus unaccompanied by the division of the cell. Thus the first recognizable megakaryocyte arises, and by successive nuclear divisions transforms itself into successive forms, each with double the preceding number of nuclei. Such an interpretation finds its confirmation also in the fact that the proportion of dividing cells and of mitotic stages are very much the same as in other nucleated marrow cells prepared by the same method (Japa, 1942).

Simultaneously with the increase of the number of nuclei in the megakaryocyte its cytoplasm develops to considerable dimensions. After attaining a certain size the cell shows signs of the degeneration which results in condensation of the nuclei, and in their closer aggregation, perhaps actual fusion in some cells, giving the appearance of a uninuclear cell. The process of degeneration in normal marrow starts in cells which have at least 8 nuclei. Simultaneously with the nuclear changes the cytoplasm breaks into particles, and finally disappears altogether. This process was observed by many authors, who saw in it the proof of the platelet production by megakaryocytes. The rate of degeneration was very much the same in all the normal cases examined (about 36 per cent.). Working with smear films Limarzi and Schleicher (1940) found a slightly smaller proportion of degenerating forms (28 per cent.) in normal marrow.

Previous authors have attempted to base the classification of megakarocytes on the morphology of the nuclear body and the differential staining of the cytoplasm, but on the present findings the only logical one seems to be that of grouping them according to the number of the nuclei in the cell. The proportion in the respective classes of the ten normal cases examined was very similar. That the largest group comprised cells with 8 nuclei indicates that the lifetime of this form is the longest. It is probably also the stage of the most extensive function. Cells with 4 nuclei have a shorter resting stage, and in those with 2 it is still shorter. The frequency of mitosis is highest in the latter group, hence the small proportion of these cells.

The counts of megakaryocytes in aceto-carmine preparations, though only approximate, show a larger proportion of these cells in marrow than reported by other investigators. The average number found in ten normal individuals was 100 per 41 thousand nucleated cells, with comparatively small variations in the different cases. The most important point in estimating the number is probably the use of isolated particles of marrow for preparation, without the admixture of current blood cells. The proportion given recently by Limarzi and Schleicher (1940)—58·8 megakaryocytes per million nucleated cells in normal marrow—is certainly much too low.

Most interesting from the clinical point of view are the changes in the proportion of megakaryocytes in the respective classes, and the qualitative and the quantitative changes in degenerating cells in the cases of pernicious anaemia and thrombopenic purpura which were examined. The data, however, are as yet suggestive rather than conclusive. It is hoped that further investigation along this line will yield more evidence regarding the behaviour and the significance of the megakaryocytes in normal and reactive bone marrow.

SUMMARY.

1. The morphology and development of megakaryocytes in normal human marrow was studied, using aceto-carmine for fixing and staining, and the squash method of preparation.

2. It was found that the megakaryocytes are multinucleated cells which develop by repeated, normal mitotic division of their nuclei without division of the cytoplasm. All nuclei within the cell undergo division synchronously, but independently, forming separate chromosome complexes, each with its own spindle mechanism. The division results in doubling of the previous number of the nuclei within the cell. The rate of the division is about 17 per 1000, as compared with 15 per 1000 among other nucleated marrow cells.

3. Five classes of megakaryocytes were distinguished according to the number of the nuclei within the cell—those with 2, 4, 8, 16 and 32 nuclei. The proportion of cells in respective classes was estimated as follows : 2·5 per cent. cells with two nuclei, 25·5 per cent. with four, 53 per cent. with eight, 18 per cent. with sixteen, and 1 per cent. with thirty-two nuclei.

4. The index of degeneration was similar in all normal cases examined—about 36 degenerating forms per 100 megakaryocytes. The process starts in cells which have at least eight nuclei.

5. The proportion of megakaryocytes in the respective classes and the rate of degeneration was characteristically changed in a case of pernicious anaemia and one of thrombopenic purpura which were examined.

6. The proportion of magakaryocytes to the other marrow cells was calculated to be approximately 100 per 41 thousand, or 2 to 5 megakaryocytes per 1000.

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INTERNATIONAL ASSOCIATION OF MICROBIOLOGISTS: NOMENCLATURE COMMITTEE.

Appointment of Judicial Commission on Bacteriological Nomenclature.

At the Third International Congress for Microbiology held in New York City in September, 1939, a series of recommendations of the Permanent International Committee on Bacteriological Nomenclature were accepted at the Plenary Session of the Congress. The third and fourth recommendations were:

> That the Nomenclature Committee, as at present constituted, shall continue to function under the auspices of the International Association of Microbiologists as it did under the International Society for Microbiology.*

> That the International Committee shall select from its membership a Judicial Commission consisting of twelve members, exclusive of members *ex officio*, and shall designate a Chairman from the membership of the Commission. The two Permanent Secretaries of the International Committee on Bacteriological Nomenclature shall be members *ex officio* of the Judicial Commission. The Commissioners shall serve in three classes of four commissioners each for nine years, so that one class of four commissioners shall retire at every International Congress. In case of the resignation or death of any commissioner, his place shall be filled for the unexpired term by the International Committee at its next meeting.

By prompt action at and subsequent to the Congress ballots were cast in spite of war conditions by 26 of the 62 members of the Permanent Committee on Nomenclature. These ballots, when examined by the undersigned Joint Secretaries of the Permanent Committee in November, 1942, were found to have resulted in the election of the persons whose names appear below. These are grouped in the three classes specified by the Permanent Committee, those receiving the highest number of votes being placed in the nine-year class, those receiving the next highest in the six-year class, etc. Names in the classes are arranged alphabetically.

Elected for nine years (term normally expires in 1948): R. E. Buchanan (U.S.A.), A. J. Kluyver (The Netherlands), E. G. D. Murray (Canada), S. Orla Jensen (Denmark).

Elected for six years (term normally expires in 1945): J. Howard Brown (U.S.A.), A. R. Prévot (France), J. Ramsbottom (Great Britain), Th. Thjötta (Norway).

Elected for three years (term normally expired in 1942): A. Lwoff (France), E. Renaux (Belgium), A. Sordelli (Argentine), C. Stapp (Germany).

* The International Society for Microbiology was replaced in 1936 by the International Association of Microbiologists, which was formed by plenary resolution at the Second International Congress for Microbiology, London, 1936.

It has been decided to make this arrangement in the hope that some plan for taking tentative action on questions of nomenclature can be developed by those members of the Commission who can be reached under war conditions.

While no provision was made in 1939 for the contingencies that have arisen, it is felt that those elected should serve until successors are elected. Professor R. E. Buchanan has been asked to act as Chairman *pro tem.* of the Judicial Commission as there is no possibility of securing an election under the rules as adopted.

ROBERT S. BREED (Geneva, New York),
R. ST.JOHN-BROOKS (London),
Joint Permanent Secretaries, International Committee on Bacteriological Nomenclature.

NOTE BY DR. R. ST.JOHN-BROOKS, LISTER INSTITUTE, ELSTREE, HERTS.

The functions of the Judicial Commission were defined by recommendations which were adopted by the Third International Congress for Microbiology, New York, 1939, in Plenary Session (see *Report of Proceedings*, p. 35). Any questions respecting interpretation of rules or recommendations in nomenclature may be submitted in the prescribed manner to the Chairman, but bacteriologists in Great Britain and the British Empire may find it convenient to refer their problems in this field in the first instance to Dr. St.John-Brooks, who will then take further necessary action.

It is hoped that the Judicial Commission will, among other matters, now be in a position to consider the proposed Bacteriological Code of Nomenclature which, under the aegis of the Nomenclature Committee, has been further developed by Professor Buchanan and his collaborators since the last Congress for final disposition at the Fourth International Congress for Microbiology whenever that may take place.